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(54) Title: METHOD FOR PRODUCING AMIDE COMPOUNDS USING A NITRILE HYDRATASE FROM A THERMOPHILIC BACILLUS

(57) Abstract

A process for the bioconversion of a nitrile to its correpsonding amide product, particularly acrylonitrile to acrylamide which is used for forming polymers. The process uses a thermophilic bacterium having a nitrile hydratase activity that is constitutively expressed, activated by cobalt ions, stable at 60 °C, and is most active between 20 °C to 70 °C with optimum activity at 55 °C. Alternatively, the process uses the enzyme extracted from the thermophilic bacterium to convert a nitrile to its amide product. The genes encoding nitrile hydratase and amidase are described in which the former is useful for the conversion of a nitrile to its amide and the latter is useful for the conversion of an amide to its acid.

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METHOD FOR PRODUCING AMIDE COMPOUNDS USING A NITRILE HYDRATASE FROM A THERMOPHILIC BACILLUS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U. S. Provisional Application Serial No. 60/083,485 which was filed April 29, 1998.

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to a novel nitrile hydratase and the DNA encoding the nitrile hydratase, from a thermophilic *Bacillus* sp., which is constitutively expressed, activated in the presence of cobalt ions, active within a temperature range of 5°C to 70°C and a pH range of 5 to 9, and stable at elevated temperatures of 50°C to 60°C in the presence of acrylonitrile for a significant period of time. Further, the present invention relates to the use of the nitrile hydratase to produce acrylamide which is used for forming polymers.

(2) Description of Related Art

Enzymatic formation of acrylamide from acrylonitrile offers advantages over the traditional copper-catalyzed process in reduction of unwanted waste products and decreased energy input, making the process a promising example of utilization of enzymes for development of "green" processes for commodity chemical production (Ashina, Y., et al., in Industrial Applications of Immobilized Biocatalysts, A. Tanaka, T. Tosa, T. Kobayashi, eds. Marcell Dekker, N.Y. p. 91-107 (1993)). Nitrile hydratases capable of catalyzing this hydration have been found in a wide variety of bacteria (Yamada, H., et al., Biosci. Biotech. Biochem. 60:1391-1400 (1996); and Cramp, R., et al., Microbiology

143:2313-2320 (1997)). Nitto Chemical Company, Japan, has pioneered the utilization of the nitrile hydratase from *Rhodococcus rhodochrous* J1 for the production of acrylamide from acrylonitrile. The *R. rhodochrous* nitrile hydratase is a novel enzyme which contains cobalt bound at the active site. The cobalt ion containing nitrile hydratases provide more attractive catalytic features than ferric ion-containing nitrile hydratases (Nagasawa, T., et al., Appl. Microbiol. Biotechnol. 40:189-195 (1993)). Although the Nitto acrylamide process is in successful production, their bioconversion process uses immobilized non-viable whole cells in the process which is run at a temperature below 10°C and with modest acrylonitrile concentrations in order to prevent inactivation of the hydratase enzyme catalyst by the acrylonitrile substrate and to avoid product polymerization (Nagasawa, T., et al., Appl. Microbiol. Biotechnol. 40:189-195 (1993)).

U.S. Patent No. 4,001,081 to Commeyras et al describes the use of various mesophilic bacterial species from the genus *Bacteridium* (in the sense of Prévot), genus *Micrococcus* (in the sense of Bergey), genus *Bacillus* and genus *Brevibacterium* (in the sense of Bergey) for conversion of nitriles to amides. In particular, the strain R 332, a mesophilic *Bacillus* species, was cited as having nitrilasic activity. R332 was shown to have optimal growth between 20°C and 40°C (see U.S. Patent No. 5,563,053 to Takashima et al). In the nitrilasic reaction with any of the above bacteria, the pH is maintained at the pH value which is limiting for the hydrolysis of the amide to its acid. The reaction temperature is 25°C.

U.S. Patent No. 4,248,968 to Watanabe et al describes a process to convert acrylonitrile to acrylamide by using bacteria strains from either *Corynebacterium* or *Nocardia* genera. However, bacteria from genus *Bacillus*, genus *Bacteridium*, and genus

Brevibacterium were also recited as being useful. The nitrile hydratase described has high activity but has low heat resistance and is inactivated in a rather short time period at temperatures between 25°C and 30°C. Therefore, while the reactions are performed between 0° and 30°C, the reactions are preferably performed at 15°C or less.

U.S. Patent No. 4,629,700 to Prevatt et al relates to conversion of aromatic polynitriles having no hydroxyl groups to an aromatic compound having at least one cyano group and one amide group or acid group. The invention discloses *Rhodococcus* species which have nitrilase systems capable of selectively hydrolyzing various cyano groups of the polynitrile. The nitrilase systems are induced either prior to or simultaneously with conversion of the aromatic polynitrile. The conversion of a dinitrile to a nitrile amide according to the invention is performed within the temperature range of 15° to 35°C.

U.S. Patent No. 4,637,982 to Yamada et al relates to a process which uses various *Pseudomonas* species to cause the conversion of a nitrile to its corresponding amide. The process is performed under alkaline conditions at a temperature between the range of 0° to 20°C. The cells prior to use in the process are cultivated in the presence of an inducer (e.g., isobutyronitrile in the case when acrylonitrile is to be hydrated).

U.S. Patent No. 5,130,235 to Beppu et al describes isolated DNA of *Rhodococcus* sp. N-774 encoding nitrile hydratase activity and recombinant *E. coli* containing the DNA. The nitrile hydratase activity appears to require Fe^{+} ions. A method for producing the nitrile hydratase in culture medium is also disclosed.

U.S. Patent No. 5,135,858 to Yamada et al describes a *Rhodococcus rhodochrous* strain J-1. wherein a lactam induces the nitrilase activity in the cultured

strain which is then used for the conversion of a nitrile to its corresponding acid. The conversion process is performed under temperatures ranging from 5° to 50°C and within a pH between 4 and 10.

U.S. Patent No. 5,334,519 to Yamada et al describes the use of cobalt cores with *Rhodococcus rhodochrous* strain J-1 to enhance the action of the nitrile hydratase in the conversion of a nitrile to its corresponding amide. In particular, the bacteria is cultivated in culture medium containing cobalt ions and a nitrile or amide inducer to induce the nitrile hydratase. The bacteria is then used for the conversion process which is performed at a temperature between 15° to 50°C and a pH that is usually between 7 to 9.

U.S. Patent No. 5,563,053 to Takashima et al describes a process for production of amide compounds from their nitrile precursor using the thermophilic bacterium *Bacillus smithii* strain SC-J05-1. The nitrile hydratase activity is inducible when a nitrile compound or an amide compound is added to the culture medium. The useful temperature range for the conversion process is between 0° and 70°C.

The foregoing provide attractive methods for production of an amide from its corresponding nitrile, in particular acrylamide from acrylonitrile. However, utilization of many of these nitrile hydratase enzymes has been limited by the requirement that very low temperatures be used for the bioconversion conditions, which increases production costs by requiring the reactions to be cooled.

SUMMARY OF THE INVENTION

The present invention provides novel thermophilic strains of *Bacillus* sp. which have a constitutive and non-inducible nitrile hydratase activity and a relatively low amidase activity which is useful for conversion of a nitrile to an amide without

producing significant amounts of the corresponding acid. In particular, the present invention relates to the conversion of acrylonitrile to acrylamide by the thermophilic *Bacillus* sp. BR449. The nitrile hydratase of *Bacillus* sp. BR449 has high activity at temperatures between 20°C and 70°C and in the presence of relatively high concentrations of the nitrile. Importantly, the nitrile hydratase has maximal activity at 55°C and is stable at temperatures up to 60°C. The hydratase activity is stimulated by cultivating the bacterium in media supplemented with divalent metal ions, particularly cobalt ions.

The present invention also provides a process for the bioconversion of a nitrile to its corresponding amide with little production of the corresponding acid. The bioconversion process uses the nitrile hydratase activity of the novel thermophilic *Bacillus* sp. BR449. In particular, the bioconversion of acrylonitrile to acrylamide is described.

Objects

It is therefore an object of the present invention to provide thermophilic strains of bacteria such as the *Bacillus* sp. disclosed herein which produces a nitrile hydratase that is active at temperatures between 20°C to 70°C and in the presence of relatively high concentrations of the nitrile wherein the nitrile hydratase converts the nitrile to its corresponding amide. In particular, the object relates to the conversion of acrylonitrile to acrylamide.

Another object of the present invention is to provide a process for producing an amide from a nitrile using the thermophilic bacteria of the invention, which comprises subjecting the nitrile in an aqueous medium to the bacterium of the present invention which has the ability to hydrolyze the acrylonitrile to acrylamide, at a temperature ranging from 20°C to 70°C and at a pH of

about 5 to 9.

A further object of the present invention is to provide the isolated DNA from the thermophilic strain of bacterium of the present invention which encodes a nitrile hydratase, wherein the DNA is operably linked to a promoter, in a plasmid, and wherein the plasmid is stably introduced into an organism such as *E. coli* to produce a transformant. The transformant produces the nitrile hydratase which hydrolyzes a nitrile to produce its corresponding amide, at a temperature ranging from 20°C to 70°C at a pH of about 5 to 9. In particular, the object relates to the conversion of acrylonitrile to acrylamide.

An object further still of the present invention is to provide the nitrile hydratase as an isolated protein produced by the thermophilic strain of *Bacillus* or an organism transformed with the isolated DNA encoding the nitrile hydratase. The isolated protein is used to hydrolyze a nitrile to produce its corresponding amide, at a temperature ranging from 20°C to 70°C at a pH of about 5 to 9. In particular, the object relates to the conversion of acrylonitrile to acrylamide.

Thus, in view of the foregoing objects, the present invention relates to bioconversion processes that are useful for the conversion of a nitrile to its corresponding amide which is rapid and economical. In particular, the bioconversion process disclosed herein relates to the conversion of acrylonitrile to acrylamide. Other objects will become increasingly apparent by reference to the following description and the drawings.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing nitrile hydratase activity with varied acrylonitrile concentration for BR449.

Figure 2 is a graph showing pH dependence of BR449 nitrile hydratase assayed at 50°C.

Figure 3 is a graph showing nitrile hydratase activity as a function of temperature.

Figure 4 is a graph showing nitrile hydratase stability. Closed circles designate specific activity in the standard assay at 50°C after two hours incubation at the temperature indicated.

Figure 5 is a graph showing acrylamide production using whole BR449 cells in 2% acrylonitrile. The temperatures are: ■ 50°C, ● 40°C, ♦ 30°C, and ▲ 22°C.

Figure 6 is a 16S gene sequence for BR449 (SEQ ID NO:1).

Figure 7 is a comparison of the DNA sequence of the 16S rRNA gene of BR449 to the 16S rRNA gene of *Bacillus* sp13 DSM 2349 (SEQ ID NO:2).

Figure 8 is a comparison of the DNA sequence of the 16S rRNA gene of BR449 to the 16S RNA gene of *Bacillus pallidus* (SEQ ID NO:3).

Figure 9 is a comparison of the DNA sequence of the 16S rRNA gene of BR449 to the 16S rRNA gene of *Bacillus smithii* (SEQ ID NO:4).

Figure 10 is the DNA sequence of a 3.3 kb DNA fragment from BR449 (SEQ ID NO:5) encoding the alpha subunit and beta subunit of the BR449 nitrile hydratase and an amidase gene. Also identified is open reading frame ORF1.

Figure 11 is the amino acid sequence of the alpha subunit of the BR449 nitrile hydratase (SEQ ID NO:9).

Figure 12 is the amino acid sequence of the beta subunit of the BR449 nitrile hydratase (SEQ ID NO:11).

Figure 13 is the amino acid sequence of the amidase gene (SEQ ID NO:7).

Figure 14 is the amino acid sequence of ORF1

(SEQ ID NO:13).

Figure 15 is a comparison of the DNA sequence of the alpha subunit of the BR449 nitrile hydratase (SEQ ID NO:8) to the DNA sequence of alpha subunit of the *B. smithii* strain SC-J05-1 nitrile hydratase (SEQ ID NO:14). Scoring matrix: ,gap penalties: -12/-2, global alignment score: 1767.

Figure 16 is a comparison of the amino acid sequence of the alpha subunit of the BR449 nitrile hydratase (SEQ ID NO:9) to the amino acid sequence of alpha subunit of the *B. smithii* strain SC-J05-1 nitrile hydratase (SEQ ID NO:15). Scoring matrix: , gap penalties: -12/-2, global alignment score: 1318.

Figure 17 is a comparison of the DNA sequence of the beta subunit of the BR449 nitrile hydratase (SEQ ID NO:10) to the DNA sequence of alpha subunit of the *B. smithii* strain SC-J05-1 nitrile hydratase (SEQ ID NO:16). Scoring matrix: , gap penalties: -12/-2, global alignment score: 1908.

Figure 18 is a comparison of the amino acid sequence of the beta subunit of the BR449 nitrile hydratase (SEQ ID NO:11) to the amino acid sequence of alpha subunit of the *B. smithii* strain SC-J05-1 nitrile hydratase (SEQ ID NO:17). Scoring matrix: , gap penalties: -12/-2, global alignment score: 1397.

Figure 19 is the result of a homology search for the amino acid sequence of ORF1 (SEQ ID NO:13) showing a partial sequence identity to the nitrile hydratase beta subunit of *Rhodococcus rhodochrous* D83695 (SEQ ID NO:18).

Figure 20 is the sequence of the 2,645 *Pst*I/*Sal*I DNA fragment which was shown to confer nitrile hydratase activity in E coli transformed with the DNA fragment. This DNA fragment is a subset of the DNA sequence (SEQ ID NO:5) shown in Figure 10.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a thermophilic strain of *Bacillus* which has a constitutive and non-inducible nitrile hydratase activity and relatively low amidase activity which is useful for conversion of acrylonitrile to acrylamide without producing significant amounts of acrylic acid. The nitrile hydratase activity is constitutively expressed, activated by a cobalt ion, active at temperatures between 20°C to 70°C, stable at 60°C, and in the presence of relatively high concentrates of the acrylonitrile. The nitrile activity is useful for conversion of other nitrile compounds to the corresponding amide compounds.

Examples of other nitrile compounds of commercial interest which can be converted into its corresponding amide compounds are aliphatic nitriles such as n-butyronitrile, n-valeronitrile, isobutyronitrile, acetonitrile and pivalonitrile; halogen-containing nitrile compounds such as 2-chloropropionitrile; unsaturated aliphatic nitrile compounds such as crotononitrile and methacrylonitrile; hydroxynitrile compounds such as lactonitrile and mendelonitrile; aminonitrile compounds such as 2-phenylglycinonitrile; aromatic nitrile compounds such as benzonitrile and cyanopyridines; and dinitrile compounds such as malononitrile, succinonitrile and adiponitrile.

The present invention specifically relates to an isolated and purified thermophilic bacterial strain which has characteristics of a member of the genus *Bacillus* deposited as ATCC 202119. The present invention also relates to a nitrile hydratase produced by the bacterial strain deposited as ATCC 202119.

The present invention also relates to a process for producing acrylamide using a thermophilic *Bacillus* sp. wherein ATCC 202119 is an example, which comprises subjecting acrylonitrile in an aqueous medium to microorganisms having the ability to hydrolyze the acrylonitrile to produce acrylamide, at a temperature

ranging from 20°C to 70°C at a pH of about 5 to 9. The conversion of acrylonitrile to acrylamide can be achieved using a broth of intact bacterial cells, disrupted bacterial cells or enzymes contained therein, or immobilized preparations obtained by immobilizing intact bacterial cells, disrupted bacterial cells or enzymes contained therein. The bacterial cells are those of a biologically pure culture of the thermophilic *Bacillus* sp. having nitrile hydratase activity. The nitrile hydratase produced by the *Bacillus* sp. is also useful for conversion of other nitrile compounds to the corresponding amide compounds. Thus, the present invention relates to a process for the conversion of a nitrile to an amide which comprises: reacting the nitrile with a thermophilic bacterium which expresses a nitrile hydratase that is active at 20°C to 70°C; and isolating the amide.

The present invention also relates to an isolated DNA encoding an enzyme having a nitrile hydratase activity wherein the enzyme is nitrile hydratase having at least 80% identity to the DNA sequence shown in SEQ ID NO:5 wherein nucleotide positions 1606 to 2292 encode the beta subunit of the nitrile hydratase and positions 2321 to 2962 encode the alpha subunit of the hydratase. Specifically, isolated DNAs are disclosed which encode the alpha subunit of the nitrile hydratase having at least 90% identity to the amino acid sequence shown in SEQ ID NO:9 and the beta subunit of the nitrile hydratase having at least 90% identity to the amino acid sequence shown in SEQ ID NO:11.

The present invention further relates to the isolated DNAs encoding the alpha and beta subunits of the nitrile hydratase enzyme (SEQ ID NO:8 and SEQ ID NO:10, respectively) wherein each isolated DNA is operably linked to a promoter in the same plasmid or separate plasmids. Alternatively, a single isolated DNA

as shown in SEQ ID NO:5 from positions 1606 to 2662 encoding both the alpha and beta subunits in tandem wherein a single promoter is operably linked to the 5' end of the DNA at position 1606. The promoter produces a polycistronic mRNA that is translated in the bacterium into alpha and beta subunit proteins. The plasmid or plasmids encoding the alpha and beta subunits are stably introduced into an organism such as *E. coli* to produce a transformant. The cloned alpha and beta subunit genes can also be expressed from suitable expression vectors when transfected into eukaryote organisms such as yeast or plants. The transformed procaryote or eukaryote organism produces the nitrile hydratase which hydrolyzes acrylonitrile to produce acrylamide, at a temperature ranging from 20°C to 70°C at a pH of about 5 to 9. The nitrile hydratase produced by the transformed organism is also useful for conversion of other nitrile compounds to its corresponding amide compounds. Thus, the present invention relates to a process for making an organism useful for the conversion of a nitrile to an amide which comprises: providing an isolated DNA encoding the nitrile hydratase; introducing the isolated DNA into an organism; and using the organism to produce the nitrile hydratase.

The present invention further relates to a nitrile hydratase as an isolated protein produced by a thermophilic *Bacillus* sp., particularly from the *Bacillus* sp. BR449 deposited as ATCC 202119. The alpha subunit comprising the isolated nitrile hydratase has an amino acid sequence substantially similar to that shown in SEQ ID NO:9 and the beta subunit comprising the isolated nitrile hydratase has an amino acid sequence substantially similar to that shown in SEQ ID NO:11. The isolated protein is used to hydrolyze acrylonitrile to produce acrylamide, at a temperature ranging from 20°C to 70°C at a pH of about 5 to 9. The nitrile activity of the isolated protein is also useful for

conversion of other nitrile compounds to the corresponding amide compounds. Thus, the process for the conversion of a nitrile to an amide comprises: reacting the nitrile with the nitrile hydratase which is produced by *Bacillus* sp. BR449 deposited as ATCC 202119 which is active at 20°C to 70°C; and optionally isolating the amide. The amide is usually not isolated. A distinguishing, novel and useful characteristic of the present invention is that the nitrile hydratase operates at higher temperatures than other nitrile hydratases in the prior art at neutral pH's.

Finally, the present invention relates to an isolated DNA having a nucleotide sequence substantially as shown in SEQ ID NO:6 encoding the amidase gene from BR449 wherein an amidase is produced having the amino acid sequence substantially as shown in SEQ ID NO:7, and an isolated DNA having a nucleotide sequence substantially as shown in SEQ ID NO:12 encoding a protein having an amino acid sequence substantially as shown in SEQ ID NO:13.

It is appropriate to recite the following embodiments for the present invention. The present invention specifically relates to the following isolated DNA embodiments. An isolated DNA encoding a nitrile hydratase consisting of an alpha and a beta subunit wherein the hydratase is optimally active at 55°C, stable at 60°C, and cobalt-containing which is useful for conversion of a nitrile to its corresponding amide without producing significant amounts of its corresponding acid. An isolated DNA wherein the nitrile hydratase has a DNA sequence as set forth in SEQ ID NO:5 wherein the sequence between positions 2312 to 2962 encodes the alpha subunit and the sequence between positions 1606 and 2292 encodes the beta subunit. An isolated DNA encoding a nitrile hydratase wherein the DNA has a nucleotide sequence which has at least 80% identity to the nucleotide sequence between positions

1601 and 2962 as set forth in SEQ ID NO:5. An isolated DNA encoding an alpha subunit of a nitrile hydratase wherein the DNA has a nucleotide sequence which has at least 80% sequence identity to the nucleotide sequence set forth in SEQ ID NO:8 and an isolated DNA encoding a beta subunit of a nitrile hydratase wherein the DNA has a nucleotide sequence which has at least 80% sequence identity to the nucleotide sequence set forth in SEQ ID NO:10. An isolated DNA encoding an amidase wherein the DNA has a nucleotide sequence which has at least 90% sequence identity to the nucleotide sequence set forth between positions 432 and 1475 in SEQ ID NO:5.

The present invention relates to the following thermophilic bacteria embodiments. A thermophilic *Bacillus* sp. having a nitrile hydratase activity that is activated by a cobalt ion which is useful for conversion of a nitrile to an amide without producing significant amounts of an acid. The thermophilic bacterial strain deposited as ATCC 202119. The thermophilic bacterial strain wherein the nitrile is acrylonitrile, the amide is acrylamide and the acid is acrylic acid.

The bacterial embodiments also include a thermophilic *Bacillus* sp. having a nitrile hydratase enzyme that is constitutively expressed, optimally active at 55°C, stable at 60°C, and cobalt-containing which is useful for conversion of a nitrile to its corresponding amide without producing significant amounts of its corresponding acid. The *Bacillus* sp. wherein the nitrile is acrylonitrile, the amide is acrylamide, and the acid is acrylic acid. The *Bacillus* sp. wherein the *Bacillus* sp. is deposited as ATCC 202119. The thermophilic *Bacillus* sp. deposited as ATCC 202119 encodes an amidase activity.

The present invention relates to the following embodiments of processes for converting a nitrile to an amide. A process for conversion of a nitrile to an amide which comprises:(a) reacting the nitrile with a

nitrile hydratase which is constitutively produced by a thermophilic *Bacillus* sp. wherein the nitrile hydratase is active at a temperature between 20°C to 70°C and active in the presence of a cobalt ion; and (b) isolating the amide. The process wherein the nitrile is acrylonitrile and the amide is acrylamide. The process wherein the reaction is conducted at a temperature within the range of 20°C to 70°C. The process wherein the nitrile hydratase is produced by the thermophilic bacterium and wherein the bacterium is deposited as ATCC 202119.

A second process embodiment, is a process for conversion of a nitrile to an amide by the action of a microorganism, the improvement comprises: (a) reacting the nitrile with a nitrile hydratase which is produced by a thermophilic *Bacillus* sp. which has been cultured in the presence of cobalt ion contained in a culture medium in an amount of about 5 to 20 mg/l and at a temperature of 60°C, to produce the nitrile hydratase in the *Bacillus* sp. which is active at 20°C to 70°C; and (b) isolating the amide produced. The process wherein the nitrile is acrylonitrile and the amide is acrylamide. The process wherein the reaction is conducted at a temperature between 20°C and 70°C. The process wherein the *Bacillus* sp. is deposited as ATCC 202119.

A third process embodiment, a process for conversion of a nitrile to an amide which comprises: (a) cultivating a thermophilic *Bacillus* sp. which has a nitrile hydratase activity that is active within the temperature range of 20°C to 70°C in a medium containing a cobalt ion; (b) making a suspension of the thermophilic *Bacillus* sp.; (c) reacting the nitrile with the suspension of thermophilic bacteria; and (b) isolating the amide. The process wherein the nitrile is acrylonitrile and the amide is acrylamide. The process wherein the reaction is conducted within a temperature range of 20°C and 70°. The process wherein the nitrile

hydratase is produced by the *Bacillus* sp. deposited as ATCC 202119.

The present invention also relates to an embodiment which is a process for conversion of a amide to an acid by the action of a microorganism, the improvement comprises: (a) reacting the amide with an amidase which is produced by a thermophilic *Bacillus* sp. deposited as ATCC 202119 which has been cultured to produce the amidase; and (b) isolating the acid produced. The process wherein the amide is acrylamide and the acid is acrylic acid. The process wherein the *Bacillus* sp. is deposited as ATCC 202119.

The present invention also embodies a nitrile hydratase produced by a bacteria strain deposited as ATCC 202119. An enzyme having nitrile hydratase activity comprising an alpha subunit which has an amino acid sequence with at least a 90% sequence identity to the sequence in SEQ ID NO:9 and a beta subunit which has an amino acid sequence with at least a 90% sequence identity to the sequence in SEQ ID NO:11.

The present invention embodies a recombinant organism comprising an isolated DNA from a bacterium deposited as ATCC 202119 wherein the DNA encodes alpha and beta subunits of a nitrile hydratase. A recombinant organism comprising an isolated DNA from a bacterium deposited as ATCC 202119 wherein the DNA encodes an amidase having 90% identity to the amino acid sequence as set forth in SEQ ID NO:7.

The present invention is not to be limited to the aforementioned embodiments.

The nitrile hydratase of the present invention is from a thermophilic *Bacillus* sp. which provides an enzyme catalyst with improved resistance to acrylonitrile inactivation. The improved resistance to inactivation allows the use of increased temperatures for production of acrylamide. The advantages of reactions held at higher temperatures include increased

reaction rates, higher solubility of the acrylamide product, and decreased cooling costs for this exothermic reaction. The isolation and properties of a preferred moderately thermophilic *Bacillus* sp. isolate which produces a thermostable nitrile hydratase is described.

Thermostable nitrile hydratases from aerobic moderate thermophiles were screened for ability to convert acrylonitrile to acrylamide at elevated temperatures and at acrylonitrile concentrations greater than 1%. A new *Bacillus* sp. (BR449) was discovered which constitutively expresses a thermostable nitrile hydratase having among its properties low substrate inhibition and optimal activity at 55°C. The nitrile hydratase cannot be induced to higher levels by exposing the cells to a nitrile, thus the nitrile hydratase expression is constitutive. The constitutive and non-inducible property of the BR449 nitrile hydratase was an unexpected property of the enzyme which was surprising in view of the prior art which discloses nitrile hydratase enzymes which are inducible. Following prolonged exposure to acrylonitrile, the BR449 nitrile hydratase exhibited a temperature-dependent inactivation by the acrylonitrile, which is attributed to alkylation of the nucleophilic sites on the enzyme.

Bacillus sp. BR449 was deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 on April 30, 1998 under the Budapest Treaty as ATCC 202119. All rights to access to the deposited strain are removed upon granting a patent on this invention. It is understood that the process of the present invention is not limited to the deposited BR449 since variants of the strain, i.e., mutants, cell fusion strains or recombinant bacteria strains derived from BR449, can also be used in the process of the instant invention. Examples of such mutants are mutant bacteria that do not express the amidase gene at all and mutants that express

the amidase gene but not the genes encoding the nitrile hydratase activity. These mutants can be related bacteria isolates that have either arisen spontaneously or under selection conditions designed to isolate the mutants. Alternatively, recombinant bacteria which selectively express either the amidase gene or the nitrile hydratase genes can constructed using any of the genetic engineering methods that are well known to those skilled in the art. Mutant or recombinant bacteria having no residual amidase activity will convert all of a nitrile substrate to its amide without production of any of the corresponding acid as a by-product. Conversely, mutant or recombinant bacteria that express the amidase gene to the exclusion of the nitrile hydratase gene are useful for converting amides to the corresponding acids.

In practicing the process of the present invention, BR449 is cultured for 2 to 3 days in culture medium containing a carbon source such as glucose. An example of a suitable medium is OP medium which is set forth in Example 1. During cultivation, it is preferable to include at least one divalent metal salt at a concentration of approximately 20 mg/l in the growth medium. The preferred divalent metal salt is Co^{2+}. After cultivation, the cells are collected from the culture medium by centrifugation and the pelleted cells resuspended in a buffer (e.g., potassium phosphate buffer) containing acrylonitrile.

The nitrile hydratase reaction is conducted in an aqueous suspension containing about 1 to 20 mg (dry weight) cells/ml and acrylonitrile between 1% and 5% by weight, preferably around 1%, in a potassium phosphate buffer at a pH between 6 and 9, preferably around 7.5. The reaction temperature is between 20°C and 70°C with the preferred temperature at 22°C which maximizes the yield of acrylamide from acrylonitrile by prolonging the lifetime of the catalyst. However, at a temperature of

55°C the nitrile hydratase activity is at a maximum but with a shorter half-life because of alkylation by of the nucleophilic sites on the enzyme. Therefore, the choice as to whether the reaction is conducted at 22°C or 55°C, or at a temperature in between, will depend on which is more important to the practitioner, nitrile hydratase reaction speed or longevity. Additionally, during the reaction, the concentration of acrylonitrile can be maintained at a preferred concentration by subsequently adding acrylonitrile to the reaction to replace the acrylonitrile that has been converted to acrylamide. The reaction is allowed to proceed for approximately two hours and the acrylamide formed during the reaction is recovered by art-known methods.

For example, the BR449 cells can be separated from the reaction mixture by centrifugation, followed by treatment with activated charcoal or an ion exchange resin to remove contaminants. Then, the amide compound can be concentrated or precipitated by distillation or evaporation under reduced pressure. Though further purification of the amide is usually not performed, the precipitated amide crystals can be recrystallized from an organic solvent such as chloroform or methanol to give the purified amide compound.

While the present invention can be practiced with intact cells, it can, from the standpoint of repeated use, continuous operation and product recovery, be preferable to immobilize the cells. Any method of immobilizing cells which is known in the art and which does not substantially reduce the nitrile hydratase activity can be used. Preferred methods include immobilization in agarose, alginate, and polyacrylamide. Examples of techniques for cell immobilization and use of immobilized cells in manufacture of amides have been described in U.S. Patent No. 4,248,968 to Watanabe et al which is herein incorporated by reference.

Cell immobilization can be accomplished by

suspending the BR449 cells in a suitable aqueous medium such as physiological saline or buffered solution containing an acrylamide monomer and cross-linking agent, then adding a suitable polymerization initiator and polymerization accelerator to the suspension and allowing the polymerization to proceed.

The acrylamide monomers used to immobilize the BR449 cells include, for example, acrylamide, methacrylamide, etc. The concentration of each monomer in the reaction should be sufficient to form a gel as a result of the polymerization reaction, and is usually between 2 and 30% by weight, based on the reaction solution. The cross-linking agents include N,N'-methylenebisacrylamide, 1,3-di-(acrylaminomethyl)-2-imidazolidone, etc. Ammonium persulfate or potassium persulfate are examples of polymerization initiators suitable for the reaction. Dimethylaminopropionitrile and triethanolamine are examples of polymerization accelerators suitable for the reaction. Any polymerization initiator or accelerator which minimally inhibits the activity of microorganisms is suitable.

Thus, there can be obtained polymer gels containing the BR449 cells which are immobilized. The gel so obtained can be crushed and used in batch method to convert acrylonitrile to acrylamide. Alternatively, the crushed gel can be packed into a column and acrylonitrile bearing solution is pumped through the column wherein the acrylonitrile is converted to acrylamide. The immobilized cells as described above can be used in a continuous column procedure.

The continuous column method uses one or a plurality of columns connected to each other in series, which are filled with the immobilized BR449 cells. The immobilized cells are crushed to a size within the range of 0.5 to 5 mm and packed into the columns at a density between 0.3 to 0.5 g immobilized cells/cc. Then, the nitrile such as acrylonitrile is continuously fed into

the column as an aqueous solution via the column inlet and, at the same time, continuously feeding the nitrile at an intermediate stage or location before completion of the reaction in an amount soluble in the reaction solution. In situations where one column is needed or used, a sectional column having one or more feed inlets provided between the column inlet and column outlet, and which comprises a few sections is preferable.

In addition to columns consisting of immobilized cells or batches containing free or immobilized cells, batches or columns containing crude or purified nitrile hydratase enzyme preparations can also be used. The advantage over using cells, either whole or immobilized, is that a much higher specific activity per unit volume or area can be achieved using enzyme extracts as opposed to intact cells. The cost of isolating BR449 nitrile hydratase can be made extremely cost effective by cloning the nitrile hydratase genes in an organism such as *E. coli* using high copy number or high expression plasmid vectors to produce the nitrile hydratase. In this manner, quantities of nitrile hydratase can be produced that are much higher than the quantities that are obtainable using BR449 cells to isolate the nitrile hydratase enzyme.

As mentioned above, the isolated DNA encoding nitrile hydratase can be isolated from BR449 and the genes encoding the alpha and beta subunits cloned into a suitable organism. The advantages for cloning the nitrile hydratase gene of BR449 into another organism are many-fold. The first advantage is that organism contains many copies of the gene which enables each organism to produce more of the enzyme compared to the quantities produced by BR449 cells. This is useful both using the whole cell method for converting a nitrile to its corresponding amide and when the enzyme is to be purified. A second advantage is that the expression of the enzyme can be made to be inducible when the genes

are operably linked to an inducible promoter. In BR449, the nitrile hydratase is continuously expressed and is not inducible. It can be anticipated that for certain applications it can be advantageous to operably link the nitrile hydratase genes to promoters that are inducible. Such promoters can be used to limit expression to those times when expression is needed. A third advantage is that the nitrile hydratase reaction can be performed at lower temperatures which prolongs the half-life of the enzyme and is useful for some conversions. Thus, at the lower temperature, more of the amide product is produced per reaction. A novel characteristic of the enzyme is its ability to operate at higher temperatures than similar nitrile hydratases in the prior art at neutral pH's. A fourth advantage is that the nitrile hydratase can be expressed in non-procaryote organisms such as yeast or plants. In plants the nitrile hydratase expression can be directed to specific parts of the plant such as the seeds or fruiting bodies which can be a very effective means for producing the nitrile hydratase. Examples of plants that are useful for expressing the nitrile hydratase or amidase genes are crop plants such as corn or tobacco, aquatic plants such as algae, or weedy plants like *Arabidopsis thaliana*.

The molecular biology techniques that can be used to clone the BR449 nitrile hydratase genes are well-known in the art (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) is an example of an authoritative guide to molecular biology) and expression vectors suitable for expression of the genes in procaryote and/or eukaryote cells are commercially available from a large number of vendors. Transformation and transfection techniques such as cell fusion, electroporation, biolistic or conventional injection are also well-known in the art and are described in Sambrook et al (*ibid.*). Other methods

which are common specifically to plants include *Agrobacterium*-mediated transformation and biolistic injection.

An attractive and useful purpose for expressing the nitrile hydratase genes in another organism is that the expression of the alpha and beta subunits can be uncoupled from expression of the amidase gene. Thus, recombinant organisms can be made which do not express any amidase activity at all, unlike the in BR449. Conversely, recombinant organisms can also be made which express only the amidase activity without any nitrile hydratase activity. These recombinant organisms can be used to convert amides to the corresponding acid. Also, by operably linking the nitrile hydratase gene or the amidase gene to the appropriate promoter expression of the gene in the recombinant organism can be increased many-fold over expression in BR449, or made inducible so that expression of the two genes can be differentially regulated.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

This example shows the isolation and characterization of nitrile-degrading thermophilic bacteria. In particular, the BR449 isolate is described and the properties of its nitrile hydratase is provided.

MATERIALS AND METHODS

Isolation of acrylonitrile-degrading thermophiles

Nitrile-degrading thermophilic bacteria were isolated from acrylonitrile enrichments of soil samples collected from pristine and polluted locations in Michigan and incubated at 60°C. The enrichment medium (DP) was designed to provide nutrients for more fastidious organisms, but to select for organisms capable of growth using acrylonitrile as a principal

carbon source. The OP medium at pH 7.2 contained per liter: K_2HPO_4, 0.5 g; NH_4Cl, 1 g; $MgSO_4$, 20 mg; yeast extract, 0.2 g; casamino acids, 0.1 g; trace element solution, 1 ml (Barnett, J. A., et al., J. Appl. Bacteriol. **18**: 131-145 (1955)), and acrylonitrile, 2 g. Primary isolations were obtained by spreading week-old suspensions of enrichment cultures on DP plates containing 0.2% acrylonitrile followed by incubation at 60°C in sealed plates. Re-streaking of isolates at progressively higher acrylonitrile concentrations yielded thermophiles capable of growth on acrylonitrile at concentrations up to 1%.

Growth of *Bacillus* isolates

Bacillus sp. BR449 (ATCC 202119) was grown in OP medium at pH 7.2 which contained per liter: KH_2PO_4, 1 g; K_2HPO_4, 0.5 g; yeast extract, 1 g; malt extract, 1 g; peptone 2 g; glycerol, 3 g; casamino acids, 0.1 g. The isolate was grown at 60°C using turbidity to monitor culture density. Divalent metal salts were added to the growth medium where indicated in concentrations of 20 mg/l.

Nitrile hydratase and amidase assay

Nitrile hydratase activity was measured using whole cells in assays containing 20 mg (dry wt) cells, in 1 ml 0.5 M acrylonitrile and potassium phosphate buffer, containing 0.5 M acrylonitrile, pH 7.5. The reaction mixture was incubated in a water bath at 50°C with shaking for 10 minutes, and the reaction stopped by addition of 0.2 ml 2 N HCl. Acrylamide formation was measured using HPLC by injection into a Novapak C-18 reverse phase column and eluted with 1:12 acetonitrile:5mM potassium phosphate buffer, pH 2.5. Peaks were identified at 200 nm with a Waters (Newton, Massachusetts) variable wavelength detector and analyzed with reference to chemical standards. One unit of

nitrile hydratase activity is defined as the formation of one μmole acrylamide per minute. Amidase activity was measured in the same manner using 0.5 M acrylamide as substrate by measurement of acrylic acid formation.

Enzyme stability studies

For studies of nitrile hydratase stability, whole cells of BR449 were incubated at varied temperatures in 50 mM potassium phosphate buffer, pH 7.5, following which the nitrile hydratase assay was carried out at 50°C.

Thermophile identification

Thermophile isolate BR449 was identified by 16S ribosomal gene sequence using universal primers and PCR methods described by Maltseva et al (Maltseva, O., et al., Microbiology 142:1115-1122 (1996)). Amplified DNA product was sequenced at the Michigan State University Sequencing Facility, East Lansing, Michigan using an Applied Biosystems Model 173A automatic sequencer. Partial sequences were compared to Gene Bank data using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information and the Ribosomal Database Project.

RESULTS

Characteristics of thermophile isolates

Following acrylonitrile enrichment of soil samples collected from varied locations, 50 isolates were selected for their growth ability on acrylonitrile. Of these, six demonstrated good growth at acrylonitrile concentrations higher than 0.2% at 50-60°C. All six isolates grew well at 50-60°C, with no growth observed at 45°C. While similar in appearance and colony morphology, these isolates demonstrated significant differences in nitrile hydratase and amidase expression.

TABLE 1

Nitrile degrading enzymes in the new isolates

New Organisms	Nitrile Hydratase Specific Activity (U/mg)	Amidase Specific Activity (U/mg)
BR443	3.0	21.4
BR444	47.2	15.5
BR445	19.2	17.5
BR446	24.8	4.9
BR447	1.1	7.0
BR448	0.8	14.6
BR449	77.6	11.3

Of these, isolate BR449 demonstrated the highest nitrile hydratase activity with only modest amidase activity, and was selected for detailed study (Table 1). This isolate could readily grow on plates and in liquid culture in 1% acrylonitrile, a concentration toxic to most other bacteria. In addition, the hydratase of BR449 proved to be quite resistant to acrylonitrile substrate inhibition, with only 47% inhibition in the presence of 10% acrylonitrile during assay at 50°C (Figure 1).

Identification of BR449

The genus/species of BR449 was determined by comparing its 16S ribosomal (rRNA) gene sequence to the 16S rRNA gene sequence of other bacteria. PCR using universal primers and PCR methods described by Maltseva et al., Microbiology **142**: 1115-1122 (1996) was used to isolate a 1.4 kb PCR product which encoded residues of the BR449 16S rRNA gene. The 1.4 kb PCR product was sequenced at the Michigan State University Sequencing Facility using an Applied Biosystems Model 173A automatic sequencer and partial DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology

Information (NCBI) and the Ribosomal Database Project. The sequences producing significant alignments are shown in Table 2.

Table 2

Sequences producing significant alignments			
Identification No.	Description	(Bits)	Value
emb|Z26929|BS16SRRNA	Bacillus sp. gene for 16S ribosomal RNA	997	0.0
emb|Z26930|BP16SRRNA	B.pallidus gene for 16S ribosomal RNA	975	0.0
emb|Z26931|BT16SRRNC	B.thermoalkalophilus gene for 16S ribosoma...	888	0.0
gb|U59630|BSU59630	Bacillus ICPS6 16S ribosomal RNA gene, seque...	720	0.0
gb|L09227|SAHRDGX	Saccharococcus thermophilus 16S ribosomal RNA...	718	0.0
gb|L29507|BAC1RRAAA	Bacillus sp. 16S ribsomal RNA (16S rRNA) gene.	714	0.0
gb|AF067651|AF067651	Bacillus caldoxylolyticus 16S ribosomal RN...	702	0.0
emb|Z26926|BT16SRRNG	B.thermocatenulatus gene for 16S ribosomal...	678	0.0
emb|X62178|BANCIMB	B.aminovorans NCIMB 8292 (T) rRNA	672	0.0
emb|Z26922|BC16SRRNB	B.caldotenax gene for 16S ribosomal RNA	668	0.0
gb|M77485|BACRRSSB	Bacillus caldovelox (DSM 411) ribosomal RNA ...	668	0.0
emb|Z26923|BT16SRRNF	B.thermoleovorans gene for 16S ribosomal RNA	664	0.0
gb|M77484|BACRRSSA	Bacillus caldolyticus (DSM 405) ribosomal RN...	664	0.0
emb|Z26924|BC16SRRNC	B.caldolyticus gene for 16S ribosomal RNA	662	0.0
emb|X60618|BKAU16S	B.kaustophilus 16S ribosomal RNA	656	0.0
emb|Z26927|BD16SRRNB	B.denitrificans gene for 16S ribosomal RNA	656	0.0
emb|Z26928|BT16SRRNH	B.thermodenitrificans gene for 16S ribosom...	654	0.0
gb|M77487|BACRRSSD	Bacillus thermodenitrificans (NCIMB 11730) r...	652	0.0
gb|M77488|BACRRSSE	Bacillus thermoleovorans (ATCC 43513) riboso...	644	0.0
emb|Z26925|BC16SRRND	B.caldovelox gene for 16S ribosomal RNA	636	0.0
emb|X60641|BTHER16SR	B.thermoglucosadicus 16S ribosomal RNA	636	0.0
emb|AJ011362|UBA011362	uncultured bacterium 16S rRNA gene, part...	630	e-179
emb|X57309|BS16SRNA	B.stearothermophilus 16S rRNA	628	e-178
emb|X62180|BCDSMRRNA	B.caldotenax DSM 406(T) rRNA	618	e-175
gb|AF078814|AF078814	Bacillus sp. 4830 16S ribosomal RNA gene, ...	593	e-167
gb|AF001964|BFAF001964	Bacillus flavothermus isolate AB005 16S ...	581	e-164
gb|AF001961|BFAF001961	Bacillus flavothermus isolate AB002 16S ...	581	e-164
emb|Z26932|BF16SRRNA	B.flavothermus gene for 16S ribosomal RNA	549	e-154
gb|AF001963|BFAF001963	Bacillus flavothermus isolate AB004 16S ...	543	e-152
emb|Z26935|BS16SRRNC	B.smithii gene for 16S ribosomal RNA	541	e-152
gb|AF001962|BFAF001962	Bacillus flavothermus isolate AB003 16S ...	537	e-151
gb|U46747|BOU46747	Bacillus sp. OS-ac-18 16S ribosomal RNA gene...	533	e-149
emb|X64465|BM16SRRN	B.methanolicus 16S ribosomal RNA	509	e-142
emb|X60643|BSMI16SR	B.smithii 16S ribosomal RNA	507	e-142
emb|Z99104|BSUB0001	Bacillus subtilis complete genome (section ...	494	e-138
dbj|D26185|BAC180K	B. subtilis DNA, 180 kilobase region of repl...	494	e-138
dbj|AB020193|AB020193	Bacillus sp. DNA for 16S ribosomal RNA, s...	494	e-138
gb|K00637|BACRGRRNB	B.subtilis rrnB operon with 23S rRNA, 16SrR...	494	e-138
emb|X94558|HL16SRRN1	H.litoralis 16S rRNA gene	494	e-138

The BR449 16S rRNA gene sequence (SEQ ID NO:1) shown in Figure 6 showed 100% identity to the 16S rRNA gene sequence of *Bacillus* sp13 (SEQ ID NO:2) as shown in Figure 7. When the 16S RNA gene sequence from BR449 was compared to the 16S rRNA gene sequence of *Bacillus pallidus* (SEQ ID NO:3), an identity of 99.6% was shown (Figure 8). Finally, the 16S RNA gene sequence from BR449 was compared to the 16S rRNA gene sequence of *Bacillus smithii* (SEQ ID NO:3). As shown in Figure 9 BR449 showed an identity of only 91.7%.

These results indicate that BR449 is not closely related to *Bacillus smithii*, but is a *Bacillus* strain which is very closely related to *Bacillus pallidus*, and virtually indistinguishable from *Bacillus* sp13 based on 16S RNA comparisons. The 100% identity of BR449 to *Bacillus* sp. is based on 16S RNA comparisons and there may be other genomic sequences which would show that BR449 is also distinct from *Bacillus* sp13. Although *Bacillus* sp13 has not been validly described, it is differentiated from other members of the moderate thermophile group by lack of extracellular amidase activity (Rainey, F.A., et al., FEMS Microbiol. Lett. 115: 205-212 (1994)). BR449 also does not show extracellular amidase activity, which is consistent with the 16S rRNA gene sequence comparisons and further indicates that BR449 is more related to *Bacillus* sp13 and *Bacillus pallidus* than to *B. smithii*.

Effect of additions on nitrile hydratase activity

Divalent metal ions were added during growth of isolate BR449 to provide indications of metals stimulatory to nitrile hydratase production. As shown in Table 3, addition of divalent cobalt greatly increased the specific activity of nitrile hydratase expressed by the cells.

TABLE 3

Effect of metal ions on nitrile hydratase activity

Metal Ions	Total Cells (mt/l, dry weight)	Nitrile hydratase Specific Activity (U/mg)	Amidase Specific Activity (U/mg)
None	90	3.2	1.8
Co^{2+}	225	71.5	9.7
Cu^{2+}	90	1.9	1.5
Fe^{2+}	490	4.3	2.3
Mn^{2+}	65	2.2	2.1
Ni^{2+}	80	6.8	3.4

This finding indicates that the BR449 nitrile hydratase is a cobalt-containing enzyme family which includes the *Rhodococcus rhodochrous* J1 and *Pseudomonas putida* NRRL-18668 nitrile hydratases (Nagasawa, T., et al., Appl. Microbiol. Biotechnol. **40**:189-195 (1993); and Payne, M.S., et al., Biochemistry **36**:5447-5454 (1997)). Induction of hydratase activity by addition of acrylonitrile during BR449 growth was without effect indicating constitutive enzyme production by the isolate. No organic acids were necessary to preserve enzyme stability.

Temperature and pH optimum of BR449

BR449 showed a broad pH optimum for activity, with a maximum near pH 7.5 (Figure 2). The BR449 nitrile hydratase has a surprisingly broad temperature range between 20° and 70°C, with an optimum at 55°C. In separate enzyme stability studies, the BR449 nitrile hydratase proved stable during two hour incubations in buffer at temperatures to 60°C, with inactivation ensuing above this temperature (Figures 3 and 4).

Acrylamide Production

In preliminary experiments to determine rates of acrylamide production with temperature, 20 mg dry weight BR449 cells were incubated with shaking in 2% acrylonitrile, 50 mM phosphate buffer, pH 7.5. As seen in Figure 5, initial reaction rates at higher temperatures were initially rapid, but ceased after one hour or less. This proved to be due to catalyst inactivation, as dilution with fresh substrate was without effect (data not shown). Although the initial reaction was slower, lowering the reaction temperature to 22°C resulted in prolonged catalyst lifetime and increased product formation.

DISCUSSION

Aerobic thermophiles were isolated which are able to grow in acrylonitrile concentrations of 1%. *Bacillus* sp. BR449 proved of particular interest in its tolerance to acrylonitrile, as well as high nitrile hydratase activity. Stimulation of enzyme specific activity in this isolate with cobalt addition to the growth medium suggests that the BR449 nitrile hydratase is likely to be a member of the cobalt-containing family. While the BR449 hydratase resists inhibition by concentrated acrylonitrile in shorter term assay at 50 degrees, longer term incubations in 2% acrylonitrile resulted in highly temperature-dependent inactivation, even at temperatures where the enzyme was stable in buffer. Since the propensity for vinyl compounds including acrylonitrile to alkylate the nucleophilic protein residues histidine and cysteine is well established (Friedman, M., J. Am. Chem. Soc. 87:3672-3682 (1967)), inactivation was likely due to acrylonitrile alkylation of nucleophilic enzyme residues important for conformational stability and/or catalytic activity, and at enzyme locations vulnerable to attack such as the active site.

Although the determinants of enzyme stability are becoming increasingly understood (Dill, K. A., Biochemistry **29**:7133-7157 (1990)), the relationships between enzyme thermostability and resistance to chemical inactivation have been less well investigated. It is generally recognized that at the same temperature, thermostable enzymes have less flexibility than their thermolabile counterparts, and are therefore more resistant to chemical denaturants (Tombs, M. P., J. Appl. Biochem. **7**:3-24 (1985)). Studies of spontaneous deamination, a major source of enzyme inactivation at both moderate and high temperatures, have demonstrated the important influences of conformational stability and neighboring residues on rates of inactivation (reviewed in Daniel, R. M., Enz. Microb. Technol. **19**:74-79 (1996)).

Thus, although the nitrile hydratase of *Bacillus* sp. BR449 exhibits a number of attractive scientific and biotechnological attributes, it is likely that identification and site-directed replacement of the alkylation-sensitive residues can provide an even more effective catalyst for acrylonitrile industrial bioconversions at elevated temperatures. The DNA encoding the enzyme can be isolated and inserted in another bacterium such as *E. coli* for production of the enzyme.

EXAMPLE 2

A 3.3 kb DNA fragment of BR449 was sequenced and identified as containing the coding region for the beta subunit and the coding region for the alpha subunit of the BR449 nitrile hydratase. Also located on the DNA fragment is the gene encoding the amidase gene and an open reading frame 1 designated as ORF1 of unknown activity. The DNA sequence of the 3.3 kb DNA fragment is shown in Figure 10 (SEQ ID NO:5).

The amino acid sequence of the alpha subunit

encoded by SEQ ID NO:8 is shown in Figure 11. The alpha subunit is a 214 amino acid protein (SEQ ID NO:9). The amino acid sequence of the BR449 alpha subunit was compared to the 220 residue amino acid sequence of the alpha subunit of the nitrile hydratase of *Bacillus smithii* strain SC-J05-1 disclosed in U.S. Patent No. 5,563,053 to Takashima (SEQ ID NO:15). Figure 16 shows that the amino acid sequence of the BR449 alpha subunit had no more than an 87.7% identity to the amino acid sequence of the alpha subunit of *Bacillus smithii*. When the DNA sequence encoding the BR449 alpha subunit (SEQ ID NO:8) was compared to the DNA encoding the alpha subunit of *Bacillus smithii* (SEQ ID NO:14), the results showed only an 81.4% degree of identity (Figure 15). These results show that the gene sequence encoding the alpha subunit of the BR449 nitrile hydratase, while related, is distinct from the homologous gene in *Bacillus smithii*.

The amino acid sequence of the beta subunit encoded by SEQ ID NO:10 is shown in Figure 12. The beta subunit is 229 amino acids (SEQ ID NO:11). The beta subunit was compared to the 229 residue amino acid sequence of the beta subunit of the nitrile hydratase of *Bacillus smithii* strain SC-J05-1 (SEQ ID NO:17). Figure 18 shows that is no more than 82.5% identity between the amino acid sequences of the beta subunit of BR449 to *Bacillus smithii*. When the DNA sequence encoding the BR449 beta subunit (SEQ ID NO:10) was compared to the DNA encoding the beta subunit of *Bacillus smithii* (SEQ ID NO:17), the results showed only an 85.6% degree of identity (Figure 17). These results show that the gene sequence encoding the beta subunit of the BR449 nitrile hydratase, while related, is distinct from the homologous gene in *Bacillus smithii*.

The sequence for the amidase gene (SEQ ID NO:6 is shown in Figure 13.

The amino acid sequence of ORF1 encoded by SEQ

ID NO:12 is shown in Figure 14. ORF1 is 101 amino acids (SEQ ID NO:13) and a homology search shown in Figure 19 showed that ORF1 has an amino acid sequence that appears to have limited identity (score 77.6) to the amino acid sequence of the beta subunit of *Rhodococcus rhodochrous* nitrile hydratase (SEQ ID NO:18).

While the nitrile hydratase of BR449 has limited amino acid identity to the nitrile hydratase of *Bacillus smithii* (87.7% and 85.6% for the alpha and beta subunits respectively), the sequence analysis clearly shows that the nitrile hydratase gene of these two organisms are distinct. Thus, these amino acid sequence differences and the 16S RNA sequence data in Example 1 indicate that BR449 is a new and hitherto unknown species of *Bacillus*. Accordingly, the biochemical properties of the BR449 nitrile hydratase shown herein is distinct from that of other microorganisms.

EXAMPLE 3

The following Example shows the production of acrylamide from acrylonitrile using intact cells in a batch method. BR449 cells are cultured at 60°C in DP medium containing 20 mg/l Co^{2+}. After 2 to 3 days, the cells are collected from the culture medium by centrifugation.

The pelleted cells are resuspended at a concentration between 1 to 20 mg (dry weight) cells/ml in a potassium phosphate buffer at pH 7.5 containing 2% acrylonitrile. The reaction temperature is at 22°C which maximizes the yield of acrylamide from acrylonitrile by prolonging the lifetime of the catalyst. During the reaction, the cells are continuously agitated and the concentration of acrylonitrile is maintained at 2% by adding acrylonitrile dropwise to the reaction. After 2 to 2.5 hours or longer, the acrylamide is measured directly from the reaction. Alternatively, the cells can be

pelleted and the acrylamide recovered from the supernatant fraction by chloroform extraction.

EXAMPLE 4

An example for the preparation of immobilized BR449 cells for use in batch production of acrylamide from acrylonitrile is as set forth below.

Four parts of intact BR449 cells (water content about 75%) grown in the presence of Co^{2+} as described in Example 4, 0.45 parts of acrylamide, 0.05 parts of N-N'-methylenebisacrylamide, and 4 parts of physiological saline are mixed to prepare a uniform suspension. To this suspension, 0.5 parts of a 5% dimethylaminopropionitrile solution and 1 part of a 2.5% potassium persulfate solution are added. The reaction is maintained at 10°C to 15°C for 30 minutes to allow polymerization. After polymerization, the cell-containing gels are crushed and washed with physiological saline to give 10 parts of immobilized cells.

Measurement of acrylamide producing ability of immobilized BR449 cells is compared to intact BR449 cells. 0.8 part of intact cells or 2 parts of the immobilized cells are diluted with 0.05 M phosphate buffer (pH 8.0) to make 100 parts. Then, for each dilution, equal parts of the diluted solution and 2% acrylonitrile are mixed together, and allowed to react at 22°C to 60°C for 30 minutes with stirring. Production of acrylamide in each of the reaction mixtures is determined using a Novapak C-18 reverse phase HPLC column as in Example 1.

EXAMPLE 5

An example for the preparation of immobilized BR449 cells for use in column production of acrylamide from acrylonitrile is as set forth below.

Forty parts of intact BR449 cells grown in the

presence of Co^{2+} as in Example 4 is mixed with 4.5 parts acrylamide, 0.5 parts N,N'-methylenebisacrylamide and 40 parts physiological saline to prepare a uniform suspension. To this suspension is added 5 parts 5% dimethylaminopropionitrile and 10 parts of a 2.5% potassium persulfate solution. The mixture is allowed to polymerize at 10°C for 30 minutes. Afterwards, the cell-containing gels are crushed into small particles and washed with physiological saline to obtain 100 parts of the immobilized cells.

Five jacketed columns, 3 cm inside diameter, 25 cm length, are each filled with 40 g of the immobilized cells and connected to each other in series. A 1.0% to 4.5% acrylonitrile aqueous solution is allowed to flow down via the top of column one at 22°C to 60°C at a flow rate of 25 to 100 ml/hr. Thereafter, 100 parts of the eluate are mixed with 4.5 parts of acrylonitrile, and allowed to flow down from the top of column two at the same temperature and similar flow rate as column one. The eluate is collected and then applied to the top of column three and allowed to flow down column three at the same temperature and similar flow rate as column two. The eluate is collected and applied to the top of column four and allowed to flow down column four at the same temperature and similar flow rate as column three. The eluate is collected and then applied to the top of column five and allowed to flow down column five at the same temperature and similar flow rate as column four. The eluate is collected and the amount of acrylamide is determined as in Example 1.

EXAMPLE 6

The nitrile hydratase enzyme of BR449 is purified according to the procedure set forth below. This purification method is only an example and is not intended to imply that it is the only method for purifying the nitrile hydratase of BR449.

Bacillus sp. BR449 (ATCC 202119) is grown in OP medium at pH 7.2 which contained per liter: KH_2PO_4, 1g; K_2HPO_4, 0.5g; yeast extract, 1g; malt extract, 1g; peptone 2g; glycerol, 3g; casamino acids, 0.1g. The isolate is grown at 60°C using turbidity to monitor culture density. Co^{2+} is added to the growth medium at a concentration of 20 mg/l.

The BR449 cells are collected by centrifugation at 10,000 x g for 10 minutes. After washing, the cells are resuspended in 300 ml of 50 mM HEPES-KOH buffer. PH 7.2, and disrupted with a French press at 20,000 psi. The cell debris and undisrupted cells are removed by centrifugation at 10,000 x g for 30 minutes. The supernatant fraction is dialysed against four changes of 10 mM HEPES-KOH buffer, pH 7.2, at 4°C for 24 hours. Afterwards, the dialysate is passed through an anion exchange column containing DEAE-Sepharose FF (Pharmacia, Piscataway, NJ) resin equilibrated with 50 mM HEPES-KOH, pH 7.2. The nitrile hydrase is allowed to be adsorbed by the column resin. The column is then washed with 50 mM HEPES-KOH, pH 7.2, to remove unbound material. Then the column is eluted using a 0.0 M to 1.0 M potassium chloride gradient of 50 mM HEPES-KOH, pH7.2. Elution fractions are analyzed for nitrile hydratase activity as set forth below. Those fractions containing nitrile hydratase are pooled and dialyzed against four changes of 10 mM HEPES-KOH buffer, pH 7.2, at 4°C for 24 hours. The dialyzate is further purified by anion chromatography as above except that the potassium chloride gradient is from 0.2 M to 0.8 M. The fractions containing nitrile hydratase activity as set forth below are pooled and dialyzed as above. This results in a crude enzyme preparation enriched for nitrile hydratase activity.

To test enzyme preparations for nitrile hydratase activity, 0.1 ml of the crude enzyme solution is added to 1.0 ml of 0.5M acrylonitrile and potassium

phosphate buffer, containing 0.5M acrylonitrile, pH 7.5. The reaction mixture is incubated in a water bath at 50°C with shaking for 10 minutes, and the reaction is stopped by addition of 0.2 ml 2N HCl. Acrylamide formation is measured using HPLC by injection into a Novapak C-18 reverse phase column and eluted with 1:12 acetonitrile:5mM potassium phosphate buffer, pH 2.5. Peaks are identified at 200 nm with a Waters (Newton, Massachusetts) variable wavelength detector and analyzed with reference to chemical standards. One unit of nitrile hydratase activity is defined as the formation of one micromole acrylamide per minute. Amidase activity is measured in the same manner using 0.5M acrylamide as substrate by measurement of acrylic acid formation.

EXAMPLE 7

This Example is to determine whether the nitrile hydratase of BR449 can convert other nitrile compounds to its corresponding amide.

Examples of other nitrile compounds that can be converted into its corresponding amide compounds according to the present invention are aliphatic nitriles such as n-butyronitrile, n-valeronitrile, isobutyronitrile, acetonitrile and pivalonitrile; halogen-containing nitrile compounds such as 2-chloropropionitrile; unsaturated aliphatic nitrile compounds such as crotononitrile and methacrylonitrile; hydroxynitrile compounds such as lactonitrile and mendelonitrile; aminonitrile compounds such as 2-phenylglycinonitrile; aromatic nitrile compounds such as benzonitrile and cyanopyridines; and dinitrile compounds such as malononitrile, succinonitrile and adiponitrile.

Bacillus sp. BR449 (ATCC 202119) is grown in DP medium at pH 7.2 which contained per liter: KH_2PO_4, 1g; K_2HPO_4, 0.5g; yeast extract, 1g; malt extract, 1g; peptone 2g; glycerol, 3g; casamino acids, 0.1g. The

isolate is grown at 60°C using turbidity to monitor culture density. Co^{2+} is added to the growth medium at a concentration of 20 mg/l.

Nitrile hydratase activity is measured using whole cells in assays containing 20 mg (dry wt) cells, in 1 ml 0.5M acrylonitrile and potassium phosphate buffer, containing 0.5M of the appropriate nitrile, pH 7.5. The reaction mixture is incubated in a water bath at 50°C with shaking for 10 minutes, and the reaction is stopped by addition of 0.2 ml 2N HCl. Amide formation is measured using HPLC by injection into a Novapak C-18 reverse phase column and elution with 1:12 acetonitrile: 5mM potassium phosphate buffer, pH 2.5. Peaks were are identified at 200 nm with a Waters (Newton, Massachusetts) variable wavelength detector and are analyzed with reference to chemical standards.

Rate of amide production from each nitrile compound at various temperatures is determined by incubating BR449 cells grown as above with shaking in 2% of the nitrile compound, 50mM phosphate buffer, pH 7.5.

EXAMPLE 8

This example describes the DNA cloning and sequencing of the nitrile hydratase and amidase genes from BR449. Figure 10 shows the region of the BR449 genome that was subcloned into plasmids for DNA sequencing. The cloning and sequencing was performed according to the procedures in Sambrook et al (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). The plasmids were commercially available. The DNA region was shown by sequencing analysis to encode the alpha and beta subunits of the nitrile hydratase gene. The region was also found to encode a gene with amidase activity and an open reading frame with unknown activity which was designated as ORF 1.

EXAMPLE 9

This example shows that a DNA fragment containing the nitrile hydratase gene conferred hydratase activity on *Escherichia coli* DH5α transformed with the DNA fragment. The nitrile hydratase activity was found to need cobalt and low temperatures for maximum activity. However, the cloned nitrile hydratase gene was found to be much better that other nitrile hydratase genes cloned into *E. coli* such as the nitrile hydratase gene from *Rhodococcus*.

Briefly, to make the recombinant *E. coli*, a 2,645 bp *Pst*I/*Sal*I DNA fragment (Figure 20) containing part of the amidase gene, the beta and alpha subunits of the nitrile hydratase gene and ORF 1 was cloned into *E. coli* DH5α using standard molecular biology techniques as disclosed by Sambrook et al in: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). The nitrile hydratase activity was measured according to the assays in Example 1. This *Pst*I/*Sal*I DNA fragment conferred nitrile hydratase activity to the recombinant *E. coli* which in was active in the presence of cobalt ions and at low temperatures. The above methods are used to clone the amidase and ORF 1 genes into *E. coli* to express their respective gene products.

EXAMPLE 10

Expression vectors and transforming bacteria, yeast, and plants with the nitrile hydratase, amidase, and/or ORF 1 genes. As an example, the nitrile hydratase gene is cloned into an expression vector which is operably linked to a promoter at the 5' end of the gene. For expression in procaryote cells, a DNA fragment containing the beta and alpha subunits in tandem is operably linked to the 5' end of the beta subunit to produce a polycistronic mRNA which is translated into separate alpha and beta subunits. One

promoter for expression of both the alpha and beta subunits is sufficient because each gene of a polycistronic mRNA is normally translated in procaryotes. However, in eukaryotes, the second coding region of a mRNA is not normally translated. Therefore, for expression of both the beta and alpha subunits in eukaryotes, the 5' end of DNA fragments, each encoding one of the subunits is each operably linked to a promoter. Thus, expression of each subunit is separate or independent of the other. An example of a eukaryote that is useful for expression of nitrile hydratase, amidase, and/or ORF 1 is yeast. Other eukaryotes that are useful for expressing the nitrile hydratase include plants.

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the Claims attached herein.

WE CLAIM:

-1-

An isolated DNA encoding a nitrile hydratase consisting of an alpha and a beta subunit wherein the hydratase is optimally active at 55°C, stable at 60°C, and cobalt-containing which is useful for conversion of a nitrile to its corresponding amide without producing significant amounts of its corresponding acid.

-2-

The isolated DNA of Claim 1 wherein the nitrile is acrylonitrile, the amide is acrylamide, and the acid is acrylic acid.

-3-

The isolated DNA of Claim 1 wherein the nitrile hydratase has a DNA sequence as set forth in SEQ ID NO:5 wherein the sequence between positions 2312 to 2962 encodes the alpha subunit and the sequence between positions 1606 and 2292 encodes the beta subunit.

-4-

An isolated DNA encoding a nitrile hydratase wherein the DNA has a nucleotide sequence which has at least 80% identity to the nucleotide sequence between positions 1601 and 2962 as set forth in SEQ ID NO:5.

-5-

An isolated DNA encoding an alpha subunit of a nitrile hydratase wherein the DNA has a nucleotide sequence which has at least 80% sequence identity to the nucleotide sequence set forth in SEQ ID NO:8.

-41-

-6-

An isolated DNA encoding a beta subunit of a nitrile hydratase wherein the DNA has a nucleotide sequence which has at least 80% sequence identity to the nucleotide sequence set forth in SEQ ID NO:10.

-7-

An isolated DNA encoding an amidase wherein the DNA has a nucleotide sequence which has at least 80% sequence identity to the nucleotide sequence set forth between positions 432 and 1475 in SEQ ID NO:5.

-8-

A thermophilic *Bacillus* sp. having a nitrile hydratase activity that is activated by a cobalt ion which is useful for conversion of a nitrile to an amide without producing significant amounts of an acid.

-42-

-9-

The thermophilic bacterial strain of Claim 8 deposited as ATCC 202119.

-10-

The thermophilic bacterial strain of Claim 8 wherein the nitrile is acrylonitrile, the amide is acrylamide and the acid is acrylic acid.

-11-

A thermophilic *Bacillus* sp. having a nitrile hydratase enzyme that is constitutively expressed, optimally active at 55°C, stable at 60°C, and cobalt-containing which is useful for conversion of a nitrile to its corresponding amide without producing significant amounts of its corresponding acid.

-12-

The *Bacillus* sp. of Claim 11 wherein the nitrile is acrylonitrile, the amide is acrylamide, and the acid is acrylic acid.

-13-

The *Bacillus* sp. of Claim 11 or 12 wherein the *Bacillus* sp. is deposited as ATCC 202119.

-14-

A thermophilic *Bacillus* sp. encoding an amidase activity deposited as ATCC 202119.

-43-

-15-

A process for conversion of a nitrile to an amide which comprises:

(a) reacting the nitrile with a nitrile hydratase which is constitutively produced by a thermophilic *Bacillus* sp. wherein the nitrile hydratase is active at a temperature between 20°C to 70°C and active in the presence of a cobalt ion; and

(b) isolating the amide.

-16-

The process of Claim 15 wherein the nitrile is acrylonitrile and the amide is acrylamide.

-17-

The process of Claim 15 or 16 wherein the reaction is conducted at a temperature within the range of 20°C to 70°C.

-18-

The process of Claim 15 or 16 wherein the nitrile hydratase is produced by the thermophilic bacterium and wherein the bacterium is deposited as ATCC 202119.

-19-

In a process for conversion of a nitrile to an amide by the action of a microorganism, the improvement comprises:

(a) reacting the nitrile with a nitrile hydratase which is produced by a thermophilic *Bacillus* sp. which has been cultured in the presence of cobalt ion contained in a culture medium in an amount of about 5 to 20 mg/l and at a temperature of 60°C, to produce the nitrile hydratase in the *Bacillus* sp. which is active at 20°C to 70°C; and

(b) isolating the amide produced.

-20-

The process of Claim 19 wherein the nitrile is acrylonitrile and the amide is acrylamide.

-21-

The process of Claim 19 or 20 wherein the reaction is conducted at a temperature between 20°C and 70°C.

-22-

The process of Claim 19 or 20 wherein the *Bacillus* sp. is deposited as ATCC 202119.

-23-

A process for conversion of a nitrile to an amide which comprises:

(a) cultivating a thermophilic *Bacillus* sp. which has a nitrile hydratase activity that is active within the temperature range of 20°C to 70°C in a medium containing a cobalt ion;

(b) making a suspension of the thermophilic *Bacillus* sp.;

(c) reacting the nitrile with the suspension of thermophilic bacteria; and

(b) isolating the amide.

-24-

The process of Claim 23 wherein the nitrile is acrylonitrile and the amide is acrylamide.

-45-

-25-

The process of Claim 23 or 24 wherein the reaction is conducted within a temperature range of 20°C and 70°C.

-26-

The process of Claim 23 or 24 wherein the nitrile hydratase is produced by the *Bacillus* sp. deposited as ATCC 202119.

-27-

In a process for conversion of an amide to an acid by the action of a microorganism, the improvement comprises:

(a) reacting the amide with an amidase which is produced by a thermophilic *Bacillus* sp. deposited as ATCC 202119 which has been cultured to produce the amidase; and

(b) isolating the acid produced.

-28-

The process of Claim 27 wherein the amide is acrylamide and the acid is acrylic acid.

-29-

The process of Claim 27 or 28 wherein the *Bacillus* sp. is deposited as ATCC 202119.

-30-

A nitrile hydratase produced by a bacteria strain deposited as ATCC 202119.

-46-

-31-

An enzyme having nitrile hydratase activity comprising an alpha subunit which has an amino acid sequence with at least a 90% sequence identity to the sequence in SEQ ID NO:9 and a beta subunit which has an amino acid sequence with at least a 90% sequence identity to the sequence in SEQ ID NO:11.

-32-

A recombinant organism comprising an isolated DNA from a bacterium deposited as ATCC 202119 wherein the DNA encodes alpha and beta subunits of a nitrile hydratase.

-33-

A recombinant organism comprising an isolated DNA from a bacterium deposited as ATCC 202119 wherein the DNA encodes an amidase having 90% identity to the amino acid sequence as set forth in SEQ ID NO:7.

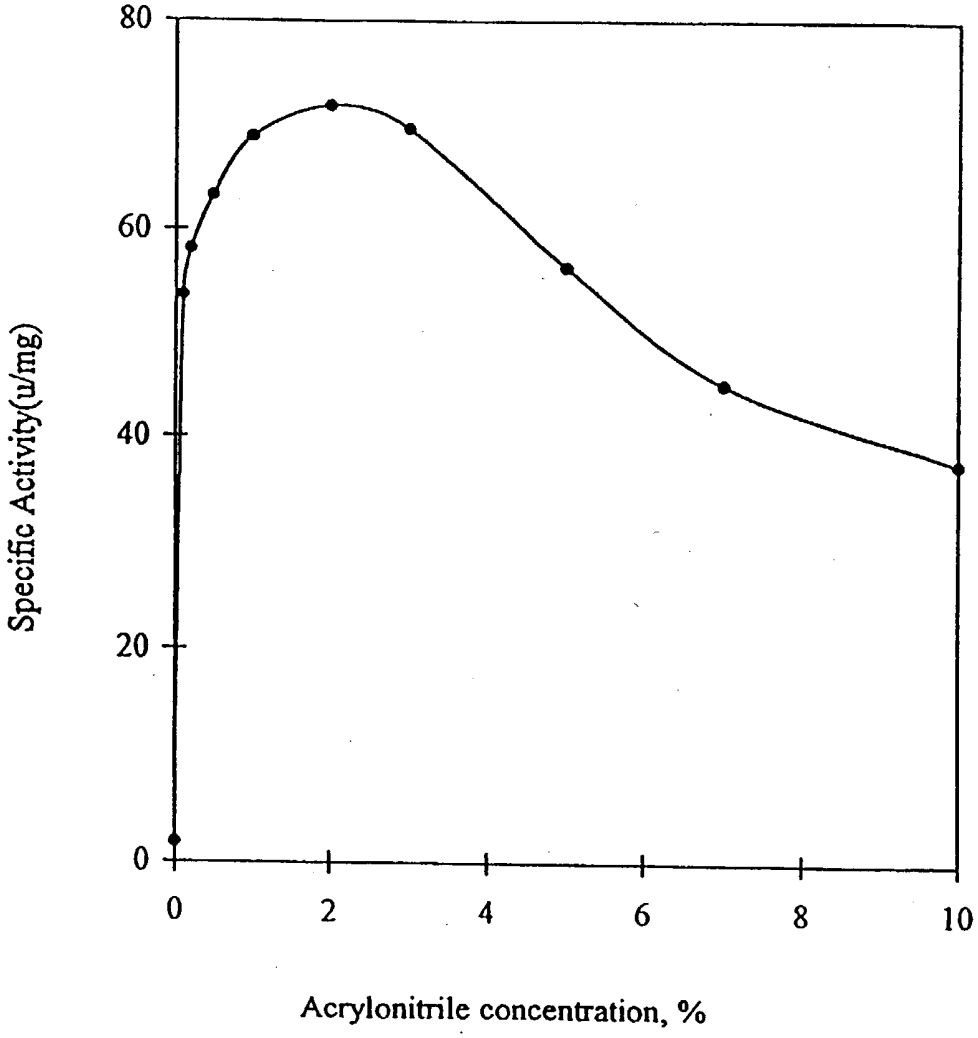
80
60
40
20
0
Specific Activity(u/mg)
0
2
4
6
8
10
Acrylonitrile concentration, %

FIG. 1

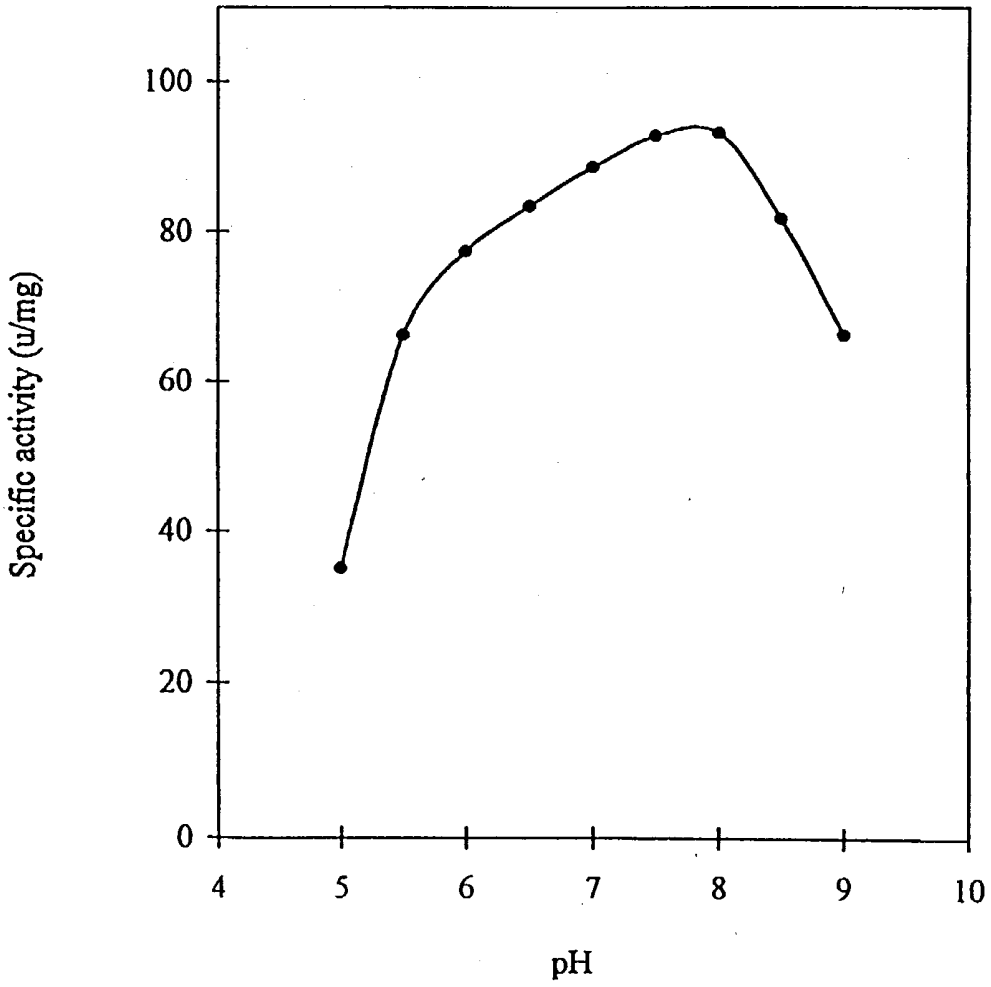

FIG. 2

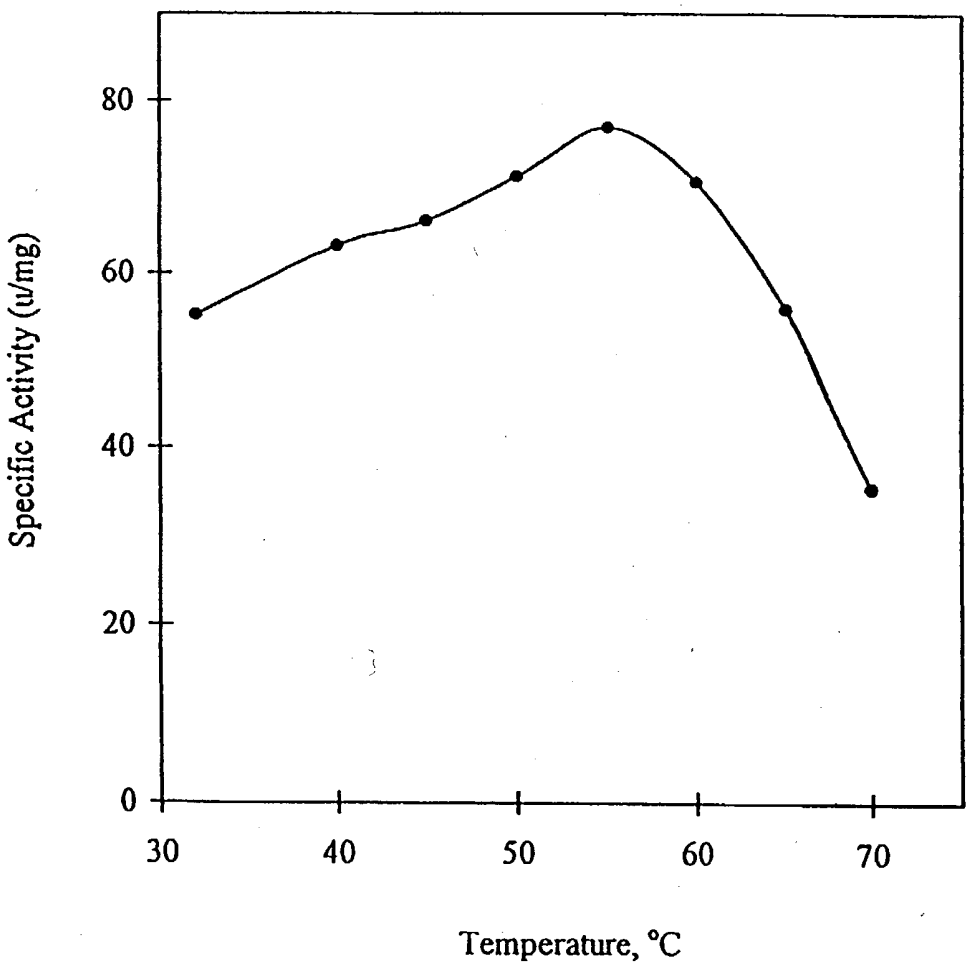
Specific Activity (u/mg)
80
60
40
20
0
30
40
50
60
70
Temperature, °C

FIG. 3

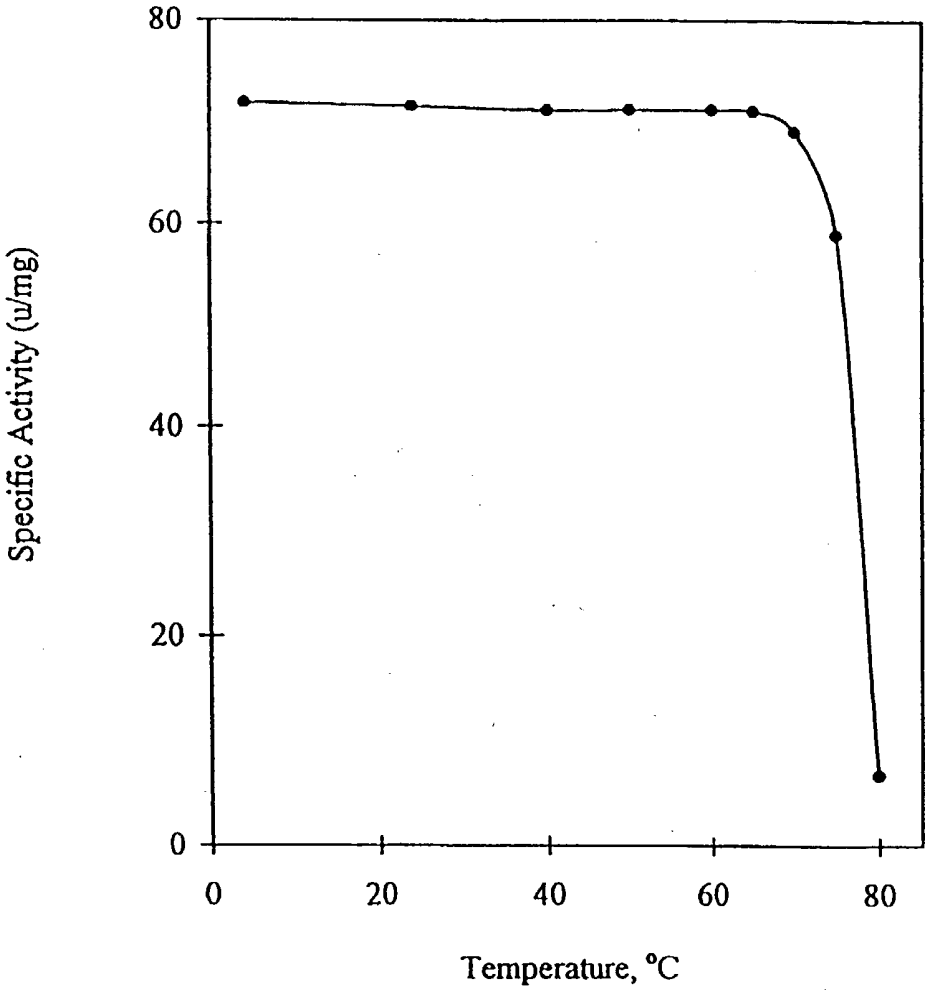

FIG. 4

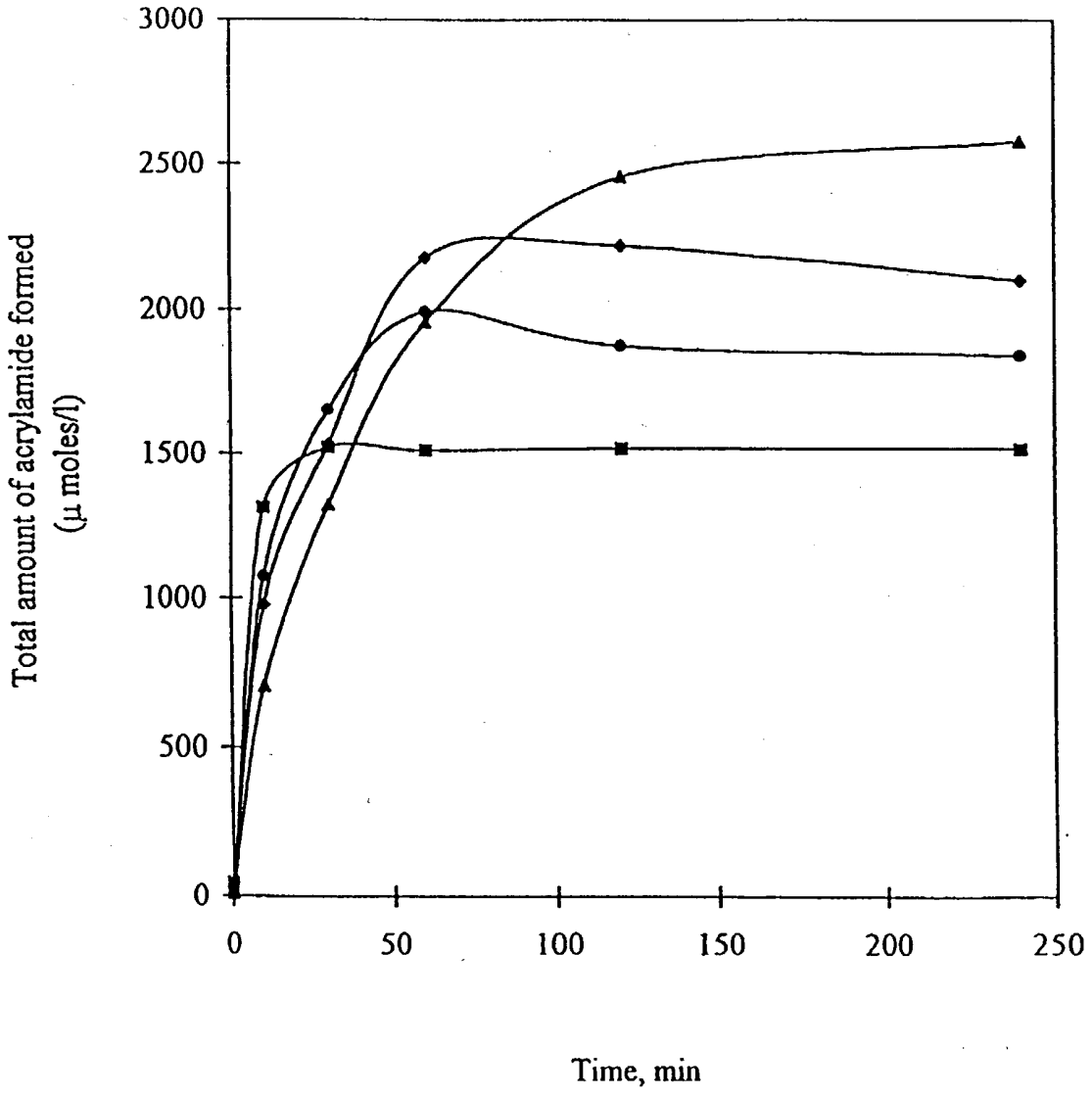
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
Total amount of acrylamide formed
(μ moles/l)
Time, min

FIG. 5

```
ctcaggacga acgctggcgg cgtgcctaat acatgcaagt cgagcggacc gaagggagct 60

tgctccttta ggttagcggc ggacgggtga gtaacacgtg ggcaacctgc cctgcagact 120

gggataactt cgggaaaccg gagctaatac cggataacac cgaaaaccgc atggttttcg 180

gttgaaaggc ggcttttagc tgtcactgca ggatgggccc gcggcgcatt agctagttgg 240

tgaggtaacg gctcaccaag gcgacgatgc gtagccgacc tgagagggtg accggccaca 300

ctgggactga gacacggccc agactcctac gggaggcagc agtagggaat cttccgcaat 360

ggacgaaagt ctgacggagc aacgccgcgt gagcgaagaa ggtcttcgga tcgtaaagct 420

ctgttgtcag ggaagaacaa gtaccgttcg aacagggcgg taccttgacg gtacctgacg 480

aggaagccac ggctaactac                                            500
```

FIG. 6

Comparison of 16S rDNA sequences: BR vs. BS

BR: BR449 16S rDNA - 500 nt
Bsp: *Bacillus sp.* 16S rDNA - 500 nt

scoring matrix: , gap penalties: -12/-2

100.0% identity; Global alignment score: 2000

```
          10        20        30        40        50        60
BR    CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGAAGGGAGCT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGAAGGGAGCT
          10        20        30        40        50        60

          70        80        90       100       110       120
BR    TGCTCCTTTAGGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCAGACT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   TGCTCCTTTAGGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCAGACT
          70        80        90       100       110       120

         130       140       150       160       170       180
BR    GGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACACCGAAAACCGCATGGTTTTCG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   GGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACACCGAAAACCGCATGGTTTTCG
         130       140       150       160       170       180

         190       200       210       220       230       240
BR    GTTGAAAGGCGGCTTTTAGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   GTTGAAAGGCGGCTTTTAGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGG
         190       200       210       220       230       240

         250       260       270       280       290       300
BR    TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACA
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACA
         250       260       270       280       290       300

         310       320       330       340       350       360
BR    CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
         310       320       330       340       350       360

         370       380       390       400       410       420
BR    GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCT
         370       380       390       400       410       420

         430       440       450       460       470       480
BR    CTGTTGTCAGGGAAGAACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Bsp   CTGTTGTCAGGGAAGAACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACG
         430       440       450       460       470       480

         490       500
BR    AGGAAGCCACGGCTAACTAC
      ::::::::::::::::::::
Bsp   AGGAAGCCACGGCTAACTAC
         490       500
```

FIG. 7

Comparison of 16S rDNA sequences: BR vs. BP

BR: BR449 16S rDNA - 500 nt
BP: *B. pallidus* 16S rDNA - 500 nt

scoring matrix: , gap penalties: -12/-2

99.6% identity; Global alignment score: 1986

```
          10        20        30        40        50        60
BR    CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGAAGGGAGCT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BP    CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGAAGGGAGCT
          10        20        30        40        50        60

          70        80        90       100       110       120
BR    TGCTCCTTTAGGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCAGACT
      :::::::::::::: ::::::::::::::::::::::::::::::::::::::::::::
BP    TGCTCCTTTAGGTTAACGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCAGACT
          70        80        90       100       110       120

         130       140       150       160       170       180
BR    GGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACACCGAAAACCGCATGGTTTTCG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BP    GGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACACCGAAAACCGCATGGTTTTCG
         130       140       150       160       170       180

         190       200       210       220       230       240
BR    GTTGAAAGGCGGCTTTTAGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGG
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BP    GTTGAAAGGCGGCTTTTAGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGG
         190       200       210       220       230       240

         250       260       270       280       290       300
BR    TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACA
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BP    TGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACA
         250       260       270       280       290       300

         310       320       330       340       350       360
BR    CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BP    CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
         310       320       330       340       350       360

         370       380       390       400       410       420
BR    GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BP    GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCT
         370       380       390       400       410       420

         430       440       450       460       470       480
BR    CTGTTGTCAGGGAAGAACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACG
      :::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::
BP    CTGTTGTCAGGGAAGAACAAGTGCCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACG
         430       440       450       460       470       480

         490       500
BR    AGGAAGCCACGGCTAACTAC
      ::::::::::::::::::::
BP    AGGAAGCCACGGCTAACTAC
         490       500
```

FIG. 8

Comparison of 16S rDNA sequences: BR vs. BS

BR: BR449 16S rDNA - 500 nt
BS: *B.smithii* 16S rDNA - 501 nt

scoring matrix: , gap penalties: -12/-2

91.7% identity; Global alignment score: 1659

```
                10        20        30        40        50
BR      CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCGA-AGGGAGC
        :::::::::::::::::::::::::::::::::::::::::::::::::::    : : :::
BS      CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACTTTCAAGAAGC
                10        20        30        40        50        60

       60        70        80        90       100       110
BR      TTGCTCCTTTAG-GTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCA-G
        :::::  :: :  :::::::::::::::::::::::::::::::::::::::: :::: :
BS      TTGCTTTTTGAAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCC-TGCAAG
                70        80        90       100       110

        120       130       140       150       160       170
BR      ACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACACCGAAAACCGCATGGTTT
        :: ::::::::: :::::::::::: ::::::::::::::: : :      ::::::
BS      ACGGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATCTTCCTTCGCATGAAGG
      120       130       140       150       160       170

        180       190       200       210       220       230
BR      TCGGTTGAAAGGCGGCTTTTAGCTGTCACT-GCAGGATGGGCCCGCGGCGCATTAGCTAG
          :::::::::::::::    ::::: : :: :::: ::::::::::::::::::::::
BS      AAGGTTGAAAGGCGGCGCA-AGCTGCCGCTTGCAG-ATGGGCCCGCGGCGCATTAGCTAG
      180       190       200       210       220       230

         240       250       260       270       280       290
BR      TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGC
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::::
BS      TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGC
        240       250       260       270       280       290

         300       310       320       330       340       350
BR      CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BS      CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
        300       310       320       330       340       350

         360       370       380       390       400       410
BR      CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAA
        :::::::::::::::::::: ::::::::::::::::::::::::::::::::::::::
BS      CAATGGACGAAAGTCTGACGGCGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAA
        360       370       380       390       400       410

         420       430       440       450       460       470
BR      AGCTCTGTTGTCAGGGAAGAACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
BS      AGCTCTGTTGTCAGGGAAGAACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCT
        420       430       440       450       460       470

         480       490       500
BR      GACGAGGAAGCCACGGCTAACTAC
        ::: :: ::::::::::::::::::
BS      GACCAGAAAGCCACGGCTAACTAC
        480       490       500
```

FIG. 9

```
TTTAACTAGGTGTTATAGGGAGAAAAAATTTATATAGGTTTACAAAAAAG
GGCATTCCTATTTATCTTTTCTACATCAATTTGAAAGGGATTATTGTGCT
TTAAATAGTGCGAATTTTCTTGAAATATTTTCGTTCTCACGTTCTATATT
TTTTACCTTTTAAAAAATCATTAATAAATGCAATCATCCTATCTTTACTT
CTTAGTCTTCAAACAGCGTGAACCACTAATAGAGCTTCTTTTAACTTTTT
CATATGATGATGTGATGCCGCCAGACATACTTAAAAACTATGCATTGATT
CATTTAGACATTCTTTAAGAGAAAATAGTTAGATTTAAAGGAGGTGATGC
CTGGGGAAATCGAACAGCAGGTCTATATATTATTATATTTAATTCACTTC
CAACATTTTATAACAAAAGGAGGAAAAAGGCATGAGACACGGGGATATTT  - amidase gene
CAAGCAGCCACGACACAGTAGGAATAGCGGTGGTCAATTACAAAATGCCG    (348 aa)
CGTTTGCACACGAAAGCAGAAGTTATTGAAAATGCAAAAAAGATCGCTGA
CATGGTCGTAGGGATGAAGCAAGGTCTTCCAGGTATGGATCTCGTCGTTT
TCCCGGAGTACAGCACAATGGGAATTATGTACGATCAGGATGAAATGTTT
GCCACTGCAGCTTCCATACCAGGAGAGGAAACAGCTATCTTTGCTGAAGC
GTGCAAAAAGGCTGATACATGGGGGGTATTCTCACTAACCGGGGAAAAAC
ATGAAGATCATCCGAATAAGGCACCATACAACACCCTAGTTCTCATTAAT
AACAAAGGAGAGATTGTGCAAAAGTACCGCAAGATTATTCCTTGGTGTCC
GATCGAAGGATGGTATCCGGGAGATACCACTTATGTCACGGAAGGACCGA
AGGGGTTGAAAATCAGTCTCATCGTTTGTGATGACGGAAATTATCCTGAA
ATCTGGCGCGATTGTGCGATGAAAGGCGCAGAATTGATCGTCCGTTGCCA
AGGCTACATGTATCCGGCAAAAGAGCAGCAAATCATGATGGCGAAAGCTA
TGGCTTGGGCGAACAATACCTATGTAGCCGTTGCCAACGCAACAGGATTT
GACGGAGTTTATTCATATTTTGGCCACTCTGCCATCATCGGTTTTGACGG
ACGCACACTAGGTGAGTGCGGAACGGAGGAGAATGGTATACAGTACGCAG
AAGTGTCCATCTCTCAGATTCGTGATTTTAGAAAGAACGCCCAGTCCCAA
AATCATTTGTTCAAGCTGCTTCACCGAGGCTATACTGGCTTGATCAACTC
CGGAGAAGGCGACCGAGGCGTAGCAGAATGCCCATTTGATTTTTATCGCA
CTTGGGTACTCGATGCAGAAAAGGCAAGAGAAAATGTAGAGAAGATCACT
AGAAGTACGGTTGGGACAGCAGAATGTCCGATTCAAGGAATCCCAAATGA
AGGAAAAACAAAAGAAATTGGTGTGTAATTCTGGAATACCAATTGTTTAA
TGCACAATAACTGCATTTTCGTCATTTTCCTTAAGTGTTAAATGAGATGA
CTAACATATGTCATCGGTAAAAATAAATTCTTAATCAAAGATGGGAGGTA
AACAAATGAACGGTATTCATGATGTTGGAGGCATGGATGGATTTGGAAAA  - beta subunit gene
GTGATGTATGTAAAAGAAGAAGAGGACATTTATTTTACACATGATTGGGA
AAGACTTGCGTTCGGACTTGTAGCTGGTTGTATGGCACAAGGATTGGGGA
TGAAGGCTTTTGATGAATTCAGGATCGGCATTGAGCTTATGCGTCCAGTG
GATTATTTGACGTCGTCGTATTATGGCCATTGGATTGCAACTGTTGCATA
CAACTTAGTAGATACGGGAGTATTAGACGAAAAAGAACTAGATGAACGAA
CGGAGGTTTTCTTGAAGAAACCTGATACCAAAATACCACGAAGAGAGGAT
CCGGCATTAGTGAAGCTTGTAGAAAAGGCACTGTATGAAGGCTTATCTCC
GATCCGTGAAATTTCAGCTTCTCCTCGGTTTAAGGTAGGAGAGAGAATCA
AGACGAAAAACATTCATCCAACTGGTCATACGAGATTCCCTCGATATGCC
CGTGACAAATATGGTGTCATTGATGAGATATATGGAGCTCATGTTTTCCC
TGATGATGCTGCTCATAGAAAAGGAGAAAACCCGCAATATCTTTACCGGG
TACGTTTTGAGGCTGAAGAATTATGGGGATATAAACAGAAAGATTCCGTT
TATATAGATCTATGGGAAAGTTATATGGAGCCTGTTTCACATTAATCATT
TTTTGAAGGAGGAATACAATATGACGATTGATCAAAAAAATACTAATATA  - alpha subunit gene
GATCCAAGATTTCCACATCATCATCCGCGTCCACAATCATTTTGGGAGGC
ACGTGCAAAAGCTCTTGAATCCTTGTTGATTGAGAAAGGGCATCTTTCCT
CAGATGCTATTGAAAGGGTAATAAAACATTATGAGCATGAGCTGGGACCA
ATGAACGGAGCAAAGGTCGTAGCGAAGGCTTGGACTGATCCTGCTTTTAA
ACAAAGATTGCTAGAAGATCCAGAGACTGTATTAAGGGAGCTAGGATACT
ATGGTTTACAGGGTGAGCATATCAGGGTAGTAGAAAATACGGATACGGTA
CACAATGTTGTAGTCTGCACTTTATGTTCATGTTACCCTTGGCCATTGCT
TGGTTTACCGCCTTCATGGTACAAAGAACCTGCTTATAGAGCTCGTGTCG
TAAAAGAGCCGAGACAAGTGTTGAAAGAATTCGGATTAGATCTTCCAGAT
TCAGTAGAAATCCGGGTATGGGACAGCAGTTCAGAAATTCGCTTTATGGT
ATTGCCGCAAAGACCTGAAGGTACGGAAGGAATGACGGAGGAGGAGCTTG
CAAAACTTGTTACTCGAGACTCCATGATTGGTGTCGCTAAAATAGAGCCG
CTAAAGTTACGGTAGGTTAGGAGGAAAATAATGAAAAGTTGTGAGAATCA  - orf1
ACCTAATGAATCATTGCTTGCGAATATGTCTGAAGAAGTCGCACCTCCTA
GAAAAAACGGAGAGTTAGAATTCCAAGAGCCTTGGGAAAGACGCTCTTTT
GGCATGACTCTTGCTTTGTACGAAGAAAAGCTGTATAGCTCTTGGGAGGA
TTTTCGATCCCGCTTGATTGAGGAGATCAAGGGGTGGGAGACCGCGAAAC
AGAAGGAGAATTCTGACTGGAACTACTATGAGCATTGGCTGGCCGCCTTG
GAACGACTAGTAGTGGAAACAGGAATGTTAAATTAAGCGTGATGTCGAC
                                                  SalI
```

FIG. 10

```
Met Thr Ile Asp Gln Lys Asn Thr Asn Ile Asp Pro Arg Phe Pro His
 1               5                  10                  15

His His Pro Arg Pro Gln Ser Phe Trp Glu Ala Arg Ala Lys Ala Leu
            20                  25                  30

Glu Ser Leu Leu Ile Glu Lys Gly His Leu Ser Ser Asp Ala Ile Glu
        35                  40                  45

Arg Val Ile Lys His Tyr Glu His Glu Leu Gly Pro Met Asn Gly Ala
    50                  55                  60

Lys Val Val Ala Lys Ala Trp Thr Asp Pro Ala Phe Lys Gln Arg Leu
65                  70                  75                  80

Leu Glu Asp Pro Glu Thr Val Leu Arg Glu Leu Gly Tyr Tyr Gly Leu
                85                  90                  95

Gln Gly Glu His Ile Arg Val Val Glu Asn Thr Asp Thr Val His Asn
            100                 105                 110

Val Val Val Cys Thr Leu Cys Ser Cys Tyr Pro Trp Pro Leu Leu Gly
        115                 120                 125

Leu Pro Pro Ser Trp Tyr Lys Glu Pro Ala Tyr Arg Ala Arg Val Val
    130                 135                 140

Lys Glu Pro Arg Gln Val Leu Lys Glu Phe Gly Leu Asp Leu Pro Asp
145                 150                 155                 160

Ser Val Glu Ile Arg Val Trp Asp Ser Ser Ser Glu Ile Arg Phe Met
                165                 170                 175

Val Leu Pro Gln Arg Pro Glu Gly Thr Glu Gly Met Thr Glu Glu Glu
            180                 185                 190

Leu Ala Lys Leu Val Thr Arg Asp Ser Met Ile Gly Val Ala Lys Ile
        195                 200                 205

Glu Pro Leu Lys Leu Arg
    210
```

FIG. 11

```
Met Asn Gly Ile His Asp Val Gly Gly Met Asp Gly Phe Gly Lys Val
 1               5                  10                  15

Met Tyr Val Lys Glu Glu Glu Asp Ile Tyr Phe Thr His Asp Trp Glu
            20                  25                  30

Arg Leu Ala Phe Gly Leu Val Ala Gly Cys Met Ala Gln Gly Leu Gly
        35                  40                  45

Met Lys Ala Phe Asp Glu Phe Arg Ile Gly Ile Glu Leu Met Arg Pro
    50                  55                  60

Val Asp Tyr Leu Thr Ser Ser Tyr Tyr Gly His Trp Ile Ala Thr Val
65                  70                  75                  80

Ala Tyr Asn Leu Val Asp Thr Gly Val Leu Asp Glu Lys Glu Leu Asp
                85                  90                  95

Glu Arg Thr Glu Val Phe Leu Lys Lys Pro Asp Thr Lys Ile Pro Arg
            100                 105                 110

Arg Glu Asp Pro Ala Leu Val Lys Leu Val Glu Lys Ala Leu Tyr Glu
        115                 120                 125

Gly Leu Ser Pro Ile Arg Glu Ile Ser Ala Ser Pro Arg Phe Lys Val
    130                 135                 140

Gly Glu Arg Ile Lys Thr Lys Asn Ile His Pro Thr Gly His Thr Arg
145                 150                 155                 160

Phe Pro Arg Tyr Ala Arg Asp Lys Tyr Gly Val Ile Asp Glu Ile Tyr
                165                 170                 175

Gly Ala His Val Phe Pro Asp Asp Ala Ala His Arg Lys Gly Glu Asn
            180                 185                 190

Pro Gln Tyr Leu Tyr Arg Val Arg Phe Glu Ala Glu Glu Leu Trp Gly
        195                 200                 205

Tyr Lys Gln Lys Asp Ser Val Tyr Ile Asp Leu Trp Glu Ser Tyr Met
    210                 215                 220

Glu Pro Val Ser His
225
```

FIG. 12

```
Met Arg His Gly Asp Ile Ser Ser Ser His Asp Thr Val Gly Ile Ala
 1               5                  10                  15

Val Val Asn Tyr Lys Met Pro Arg Leu His Thr Lys Ala Glu Val Ile
            20                  25                  30

Glu Asn Ala Lys Lys Ile Ala Asp Met Val Val Gly Met Lys Gln Gly
        35                  40                  45

Leu Pro Gly Met Asp Leu Val Val Phe Pro Glu Tyr Ser Thr Met Gly
    50                  55                  60

Ile Met Tyr Asp Gln Asp Glu Met Phe Ala Thr Ala Ala Ser Ile Pro
65                  70                  75                  80

Gly Glu Glu Thr Ala Ile Phe Ala Glu Ala Cys Lys Lys Ala Asp Thr
                85                  90                  95

Trp Gly Val Phe Ser Leu Thr Gly Glu Lys His Glu Asp His Pro Asn
            100                 105                 110

Lys Ala Pro Tyr Asn Thr Leu Val Leu Ile Asn Asn Lys Gly Glu Ile
        115                 120                 125

Val Gln Lys Tyr Arg Lys Ile Ile Pro Trp Cys Pro Ile Glu Gly Trp
    130                 135                 140

Tyr Pro Gly Asp Thr Thr Tyr Val Thr Glu Gly Pro Lys Gly Leu Lys
145                 150                 155                 160

Ile Ser Leu Ile Val Cys Asp Asp Gly Asn Tyr Pro Glu Ile Trp Arg
                165                 170                 175

Asp Cys Ala Met Lys Gly Ala Glu Leu Ile Val Arg Cys Gln Gly Tyr
            180                 185                 190

Met Tyr Pro Ala Lys Glu Gln Gln Ile Met Met Ala Lys Ala Met Ala
        195                 200                 205
```

FIG. 13A

```
Trp Ala Asn Asn Thr Tyr Val Ala Val Ala Asn Ala Thr Gly Phe Asp
    210                 215                 220

Gly Val Tyr Ser Tyr Phe Gly His Ser Ala Ile Ile Gly Phe Asp Gly
225                 230                 235                 240

Arg Thr Leu Gly Glu Cys Gly Thr Glu Glu Asn Gly Ile Gln Tyr Ala
                245                 250                 255

Glu Val Ser Ile Ser Gln Ile Arg Asp Phe Arg Lys Asn Ala Gln Ser
            260                 265                 270

Gln Asn His Leu Phe Lys Leu Leu His Arg Gly Tyr Thr Gly Leu Ile
        275                 280                 285

Asn Ser Gly Glu Gly Asp Arg Gly Val Ala Glu Cys Pro Phe Asp Phe
    290                 295                 300

Tyr Arg Thr Trp Val Leu Asp Ala Glu Lys Ala Arg Glu Asn Val Glu
305                 310                 315                 320

Lys Ile Thr Arg Ser Thr Val Gly Thr Ala Glu Cys Pro Ile Gln Gly
                325                 330                 335

Ile Pro Asn Glu Gly Lys Thr Lys Glu Ile Gly Val
            340                 345
```

FIG. 13B

```
Met Lys Ser Cys Glu Asn Gln Pro Asn Glu Ser Leu Leu Ala Asn Met
  1               5                  10                  15

Ser Glu Glu Val Ala Pro Pro Arg Lys Asn Gly Glu Leu Glu Phe Gln
            20                  25                  30

Glu Pro Trp Glu Arg Arg Ser Phe Gly Met Thr Leu Ala Leu Tyr Glu
        35                  40                  45

Glu Lys Leu Tyr Ser Ser Trp Glu Asp Phe Arg Ser Arg Leu Ile Glu
    50                  55                  60

Glu Ile Lys Gly Trp Glu Thr Ala Lys Gln Lys Glu Asn Ser Asp Trp
65                  70                  75                  80

Asn Tyr Tyr Glu His Trp Leu Ala Ala Leu Glu Arg Leu Val Val Glu
                85                  90                  95

Thr Gly Met Leu Asn
            100
```

FIG. 14

Comparison of alpha subunit genes: BR vs. BS

BR-A: BR449 (642 nt); BS-A: *B. smithii* (660 nt)

81.4% identity;

```
                10                  20          30          40
BR-A    ATGACGATTGATCAAAAAA----------ATACTAATATAGATCCAAGATTTCCACAT
        ::: : ::::: ::::::            ::  : :  : ::::: ::::::::::
BS-A    ATGGCAATTGAACAAAAATTGATGGATGATCATCATGAAGTGGATCCGCGATTTCCACAT
                10          20          30          40          50          60

        50          60          70          80          90          100
BR-A    CATCATCCGCGTCCACAATCATTTTGGGAGGCACGTGCAAAAGCTCTTGAATGCTTGTTG
        :::::::: :: :: ::::: :::::::: ::::: :: ::::: :::::::::  ::::
BS-A    CATCATCCCCGGCCGCAATCGTTTTGGGAAGCACGGGCTAAAGCGCTTGAATCTCTGTTA
                70          80          90          100         110         120

      110         120         130         140         150         160
BR-A    ATTGAGAAAGGGCATCTTTCCTCAGATGCTATTGAAAGGGTAATAAAACATTATGAGCAT
        ::::::::: : : ::::::::: :: :: ::::: ::::: :::::::: ::::: :::
BS-A    ATTGAGAAAAGACTTCTTTCCTCTGACGCCATTGAGAGGGTTATAAAACACTATGAACAT
                130         140         150         160         170         180

      170         180         190         200         210         220
BR-A    GAGCTGGGACCAATGAACGGAGCAAAGGTCGTAGCGAAGGCTTGGACTGATCCTGCTTTT
        ::::: :: :: :::::::::::: :: ::::: :::::::: ::::: :::::::  :::
BS-A    GAGCTTGGGCCGATGAACGGAGCTAAAGTCGTTGCGAAGGCCTGGACCGATCCTGAATTT
                190         200         210         220         230         240

      230         240         250         260         270         280
BR-A    AAACAAAGATTGCTAGAAGATCCAGAGACTGTATTAAGGGAGCTAGGATACTATGGTTTA
        :::::::::::::: :::::::::: ::::: ::  :::: :: ::::: : :::: :
BS-A    AAACAAAGATTGCTGGAAGATCCAGAAACTGTGTTGCGGGAACTTGGATATTTTGGTCTG
                250         260         270         280         290         300

      290         300         310         320         330         340
BR-A    CAGGGTGAGCATATCAGGGTAGTAGAAAATACGGATACGGTACACAATGTTGTAGTCTGC
        :: :: :::::::::::::::::: :::::::::::::::::::::::::::: :: :: :::
BS-A    CAAGGAGAGCATATCAGGGTAGTGGAAAATACGGATACGGTACACAATGTAGTGGTTTGC
                310         320         330         340         350         360

      350         360         370         380         390         400
BR-A    ACTTTATGTTCATGTTACCCTTGGCCATTGCTTGGTTTACCGCCTTCATGGTACAAAGAA
        ::: :::::::::::: ::::::::  :::::::::::::::::::::::::: ::::::
BS-A    ACTCTATGTTCATGTTATCCTTGGCCGCTGCTTGGTTTACCGCCTTCATGGTATAAAGAA
                370         380         390         400         410         420

      410         420         430         440         450         460
BR-A    CCTGCTTATAGAGCTCGTGTCGTAAAAGAGCCGAGACAAGTGTTGAAAGAATTCGGATTA
        :: :: ::  :  :::: :: :: :::::::::::: ::::  :: ::::::::::::::
BS-A    CCGGCCTACCGTTCTCGGGTTGTTAAAGAGCCGAGAAAAGTACTGCAAGAATTCGGATTA
                430         440         450         460         470         480

      470         480         490         500         510         520
BR-A    GATCTTCCAGATTCAGTAGAAATCCGGGTATGGGACAGCAGTTCAGAAATTCGCTTTATG
        ::  : :: ::::::::::::::: ::::: :::::::: ::::::::: :::: ::::::
BS-A    GACTTGCCGGATTCAGTAGAAATTCGGGTTTGGGACAGTAGTTCAGAAGTTCGTTTTATG
                490         500         510         520         530         540

      530         540         550         560         570         580
BR-A    GTATTGCCGCAAAGACCTGAAGGTACGGAAGGAATGACGGAGGAGGAGCTTGCAAAACTT
        :::::::::::::::::::: :: :: :::::::::::::::::::::::  ::  :: :
BS-A    GTATTGCCGCAAAGACCTGAGGGCACAGAAGGAATGACGGAGGAGGAGCTGGCGCAAATC
                550         560         570         580         590         600

      590         600         610         620         630         640
BR-A    GTTACTCGAGACTCCATGATTGGTGTCGCTAAAATAGAGCCGC-TAAAGTTACGG-----
        :::::::: :::::::::::::: ::::: ::: :  :::::: :::::: :
BS-A    GTTACTCGTGACTCCATGATTGGCGTCGCCAAAGTTCAGCCGCCTAAAGTGATCCAAGAA
                610         620         630         640         650         660
```

FIG. 15

Comparison of alpha subunit proteins: BR vs. BS

BR-B: BR449 (214 aa); BS-B: *B. smithii* (220 aa)

87.7% identity;

```
                10        20        30        40        50
BR-B    MTIDQK----NTNIDPRFPHHHPRPQSFWEARAKALESLLIEKGHLSSDAIERVIKHYEH
        :.:.::    . ..:::::::::::::::::::::::::::::  ::::::::::::::
BS-B    MAIEQKLMDDHHEVDPRFPHHHPRPQSFWEARAKALESLLIEKRLLSSDAIERVIKHYEH
                10        20        30        40        50        60

          60        70        80        90       100       110
BR-B    ELGPMNGAKVVAKAWTDPAFKQRLLEDPETVLRELGYYGLQGEHIRVVENTDTVHNVVVC
        :::::::::::::::::: ::::::::::::::::::.:::::::::::::::::::::::
BS-B    ELGPMNGAKVVAKAWTDPEFKQRLLEDPETVLRELGYFGLQGEHIRVVENTDTVHNVVVC
                70        80        90       100       110       120

         120       130       140       150       160       170
BR-B    TLCSCYPWPLLGLPPSWYKEPAYRARVVKEPRQVLKEFGLDLPDSVEIRVWDSSSEIRFM
        :::::::::::::::::::::::.:::::::.::.:::::::::::::::::::::.:::
BS-B    TLCSCYPWPLLGLPPSWYKEPAYRSRVVKEPRKVLQEFGLDLPDSVEIRVWDSSSEVRFM
                130       140       150       160       170       180

         180       190       200       210
BR-B    VLPQRPEGTEGMTEEELAKLVTRDSMIGVAKIEPLKL--R
        ::::::::::::::::::..:::::::::::..: :.  .
BS-B    VLPQRPEGTEGMTEEELAQIVTRDSMIGVAKVQPPKVIQE
                190       200       210       220
```

FIG. 16

Comparison of beta subunit genes: BR vs. BS

BR-B: BR449 (687 nt); BS-B: *B. smithii* (687 nt)

82.5% identity;

```
                10        20        30        40        50        60
BR-B    ATGAACGGTATTCATGATGTTGGAGGCATGGATGGATTTGGAAAAGTGATGTATGTAAAA
        ::::: :: ::::::::::::::: :::::::::::::::::: ::: : :::::::: :::
BS-B    ATGAATGGGATTCATGATGTTGGCGGCATGGATGGATTTGGGAAAATTATGTATGTGAAA
                10        20        30        40        50        60

                70        80        90       100       110       120
BR-B    GAAGAAGAGGACATTTATTTTACACATGATTGGGAAAGACTTGCGTTCGGACTTGTAGCT
        ::::: :: :: : :::::: : ::::::::::::::: ::: : ::::: ::::: ::
BS-B    GAAGAGGAAGATACTTATTTCAAACATGATTGGGAAAGGCTTACTTTCGGTCTTGTTGCC
                70        80        90       100       110       120

               130       140       150       160       170       180
BR-B    GGTTGTATGGCACAAGGATTGGGGATGAAGGCTTTTGATGAATTCAGGATCGGCATTGAG
        :: :: ::::: :::::::::: :::::::::::::::::::: ::::: ::::::::
BS-B    GGCTGCATGGCTCAAGGATTGGGAATGAAGGCTTTTGATGAATTTAGGATTGGCATTGAA
               130       140       150       160       170       180

               190       200       210       220       230       240
BR-B    CTTATGCGTCCAGTGGATTATTTGACGTCGTCGTATTATGGCCATTGGATTGCAACTGTT
          :::::::::: ::::::: :::: :: :: :::::::: :::::::::::::: ::
BS-B    AAAATGCGTCCAGTTGATTATCTGACATCATCCTATTATGGTCATTGGATTGCAACCGTC
               190       200       210       220       230       240

               250       260       270       280       290       300
BR-B    GCATACAACTTAGTAGATACGGGAGTATTAGACGAAAAAGAACTAGATGAACGAACGGAG
        ::::::::::::  : :: ::::::::: : :: ::::::::: : :: :: ::::::  :
BS-B    GCATACAACTTGTTGGAAACGGGAGTACTGGATGAAAAAGAATTGGAAGATCGAACACAA
               250       260       270       280       290       300

               310       320       330       340       350       360
BR-B    GTTTTCTTGAAGAAACCTGATACCAAAATACCACGAAGAGAGGATCCGGCATTAGTGAAG
        : :::: :: : ::::: :: :::::::::: ::: : ::: ::::: :::::: :::
BS-B    GCTTTCATGGAAAAACCCGACACCAAAATACAACGTTGGGAGAATCCGAAATTAGTTAAG
               310       320       330       340       350       360

               370       380       390       400       410       420
BR-B    CTTGTAGAAAAGGCACTGTATGAAGGCTTATCTCCGATCCGTGAAATTTCAGCTTCTCCT
         :::::::::: :: :::  :::::: ::::::::  :::::::: ::::  : : :::
BS-B    GTTGTAGAAAAAGCCCTGCTTGAAGGTTTATCTCCTGTCCGTGAAGTTTCCTCATTTCCA
               370       380       390       400       410       420

               430       440       450       460       470       480
BR-B    CGGTTTAAGGTAGGAGAGAGAATCAAGACGAAAAACATTCATCCAACTGGTCATACGAGA
        :::::: :::: ::::: ::::: ::::: :  :::::::: ::::: :: :: :: :::
BS-B    CGGTTTGAGGTGGGAGAAAGAATAAAGACAAGGAACATTCACCCAACAGGCCACACTAGA
               430       440       450       460       470       480

               490       500       510       520       530       540
BR-B    TTCCCTCGATATGCCCGTGACAAATATGGTGTCATTGATGAGATATATGGAGCTCATGTT
        :: :: ::::: :  :: :: :: ::::: :::::::: ::: ::::::: :::::::::
BS-B    TTTCCACGATACGTGCGCGATAAGTATGGAGTCATTGAAGAGGTATATGGGGCTCATGTT
               490       500       510       520       530       540

           550       560       570       580       590       600
BR-B    TTCCCTGATGATGCTGCTCATAGAAAAGGAGAAAACCCGCAATATCTTTACCGGGTACGT
        :::::::::::: :::::::: :::::::::::::::::::::::::::: :: :: :::::::
BS-B    TTCCCTGATGACGCTGCTCACAGAAAAGGAGAAAACCCGCAATATCTCTATCGTGTACGT
               550       560       570       580       590       600

               610       620       630       640       650       660
BR-B    TTTGAGGCTGAAGAATTATGGGGATATAAACAGAAAGATTCCGTTTATATAGATCTATGG
        ::::: :: ::::::::::::::   :::::::: ::::: :::::::: ::::: :::
BS-B    TTTGATGCCGAAGAATTATGGGGAGTAAAACAGAATGATTCAGTTTATATCGATCTTTGG
               610       620       630       640       650       660

               670       680
BR-B    GAAAGTTATATGGAGCCTGTTTCACAT
        ::: ::::: :::: ::::::::::::
BS-B    GAAGGTTATTTGGAACCTGTTTCACAT
               670       680
```

FIG. 17

Comparison of beta subunit proteins: BR vs. BS

BR-B: BR449 (229 aa); BS-B: *B. smithii* (229 aa)

85.6% identity;

```
            10        20        30        40        50        60
BR-B    MNGIHDVGGMDGFGKVMYVKEEEDIYFTHDWERLAFGLVAGCMAQGLGMKAFDEFRIGIE
        :::::::::::::::::.::::::: :: :::::::.:::::::::::::::::::::::
BS-B    MNGIHDVGGMDGFGKIMYVKEEEDTYFKHDWERLTFGLVAGCMAQGLGMKAFDEFRIGIE
            10        20        30        40        50        60

            70        80        90       100       110       120
BR-B    LMRPVDYLTSSYYGHWIATVAYNLVDTGVLDEKELDERTEVFLKKPDTKIPRREDPALVK
         :::::::::::::::::::::::..:::::::::..::..:..::::::: : :.: :::
BS-B    KMRPVDYLTSSYYGHWIATVAYNLLETGVLDEKELEDRTQAFMEKPDTKIQRWENPKLVK
            70        80        90       100       110       120

           130       140       150       160       170       180
BR-B    LVEKALYEGLSPIREISASPRFKVGERIKTKNIHPTGHTRFPRYARDKYGVIDEIYGAHV
        .:::::: ::::::.::.:. :::.::::::::.:::::::::::::::.::::::::.:.:::::
BS-B    VVEKALLEGLSPVREVSSFPRFEVGERIKTRNIHPTGHTRFPRYVRDKYGVIEEVYGAHV
           130       140       150       160       170       180

           190       200       210       220
BR-B    FPDDAAHRKGENPQYLYRVRFEAEELWGYKQKDSVYIDLWESYMEPVSH
        :::::::::::::::::::::::.:::::: ::.:::::::::.:.:::::
BS-B    FPDDAAHRKGENPQYLYRVRFDAEELWGVKQNDSVYIDLWEGYLEPVSH
           190       200       210       220
```

FIG. 18

Homology search of ORF1 (101 aa)

```
gnl|PID|d1012733 (D83695) nitrile hydratase b-subunit homolog [Rhodococcus
          rhodochrous]
          Length = 148

 Score = 77.6 bits (188), Expect = 2e-14
 Identities = 40/107 (37%), Positives = 61/107 (56%), Gaps = 9/107 (8%)

Query: 1   MKSCENQPNESLLANMSEEVAP--------PRKNGELEFQEPWERRSFGMTLALYEEKLYS 53
            M     QP+  L AN+ + V          PR++GE+ F + WE R+F +  AL+ +  +
Sbjct: 1   MPRLNEQPHPGLEANLGDLVQNLPFNERIPRRSGEVAFDQAWEIRAFSIATALHGQGRF- 59

Query: 54  SWEDFRSRLIEEIKGWETAKQKENSDWNYYEHWLAALERLVVETGML 100
            W++F+SRLIE IK WE A+       W+YYE W+ ALE L+ + G +
Sbjct: 60  EWDEFQSRLIESIKQWE-AEHATTEQWSYYERWMLALEELLHDKGFV 105
```

FIG. 19

PstI-SalI DNA fragment (2645 nt) which exhibits Nitrile Hydratase Activity in *E. coli* DH5α

PstI

CTGCAGCTTCCATACCAGGAGAGGAAACAGCTATCTTTGCTGAAGCGTGC
AAAAAGGCTGATACATGGGGGGTATTCTCACTAACCGGGGAAAAACATGA
AGATCATCCGAATAAGGCACCATACAACACCCTAGTTCTCATTAATAACA
AAGGAGAGATTGTGCAAAAGTACCGCAAGATTATTCCTTGGTGTCCGATC
GAAGGATGGTATCCGGGAGATACCACTTATGTCACGGAAGGACCGAAGGG
GTTGAAAATCAGTCTCATCGTTTGTGATGACGGAAATTATCCTGAAATCT
GGCGCGATTGTGCGATGAAAGGCGCAGAATTGATCGTCCGTTGCCAAGGC
TACATGTATCCGGCAAAAGAGCAGCAAATCATGATGGCGAAAGCTATGGC
TTGGGCGAACAATACCTATGTAGCCGTTGCCAACGCAACAGGATTTGACG
GAGTTTATTCATATTTTGGCCACTCTGCCATCATCGGTTTTGACGGACGC
ACACTAGGTGAGTGCGGAACGGAGGAGAATGGTATACAGTACGCAGAAGT
GTCCATCTCTCAGATTCGTGATTTTAGAAAGAACGCCCAGTCCCAAAATC
ATTTGTTCAAGCTGCTTCACCGAGGCTATACTGGCTTGATCAACTCCGGA
GAAGGCGACCGAGGCGTAGCAGAATGCCCATTTGATTTTTATCGCACTTG
GGTACTCGATGCAGAAAAGGCAAGAGAAAATGTAGAGAAGATCACTAGAA
GTACGGTTGGGACAGCAGAATGTCCGATTCAAGGAATCCCAAATGAAGGA
AAAACAAAAGAAATTGGTGTGTAATTCTGGAATACCAATTGTTTAATGCA
CAATAACTGCATTTTCGTCATTTTCCTTAAGTGTTAAATGAGATGACTAA
CATATGTCATCGGTAAAAATAAATTCTTAATCAAAGATGGGAGGTAAACA
AATGAACGGTATTCATGATGTTGGAGGCATGGATGGATTTGGAAAAGTGA - beta subunit gene
TGTATGTAAAAGAAGAAGAGGACATTTATTTTACACATGATTGGGAAAGA
CTTGCGTTCGGACTTGTAGCTGGTTGTATGGCACAAGGATTGGGGATGAA
GGCTTTTGATGAATTCAGGATCGGCATTGAGCTTATGCGTCCAGTGGATT
ATTTGACGTCGTCGTATTATGGCCATTGGATTGCAACTGTTGCATACAAC
TTAGTAGATACGGGAGTATTAGACGAAAAAGAACTAGATGAACGAACGGA
GGTTTTCTTGAAGAAACCTGATACCAAAATACCACGAAGAGAGGATCCGG
CATTAGTGAAGCTTGTAGAAAAGGCACTGTATGAAGGCTTATCTCCGATC
CGTGAAATTTCAGCTTCTCCTCGGTTTAAGGTAGGAGAGAGAATCAAGAC
GAAAAACATTCATCCAACTGGTCATACGAGATTCCCTCGATATGCCCGTG
ACAAATATGGTGTCATTGATGAGATATATGGAGCTCATGTTTTCCCTGAT
GATGCTGCTCATAGAAAAGGAGAAAACCCGCAATATCTTTACCGGGTACG
TTTTGAGGCTGAAGAATTATGGGGATATAAACAGAAAGATTCCGTTTATA
TAGATCTATGGGAAAGTTATATGGAGCCTGTTTCACATTAATCATTTTTT
GAAGGAGGAATACAATATGACGATTGATCAAAAAAATACTAATATAGATC - alpha subunit gene
CAAGATTTCCACATCATCATCCGCGTCCACAATCATTTTGGGAGGCACGT
GCAAAAGCTCTTGAATCCTTGTTGATTGAGAAAGGGCATCTTTCCTCAGA
TGCTATTGAAAGGGTAATAAAACATTATGAGCATGAGCTGGGACCAATGA
ACGGAGCAAAGGTCGTAGCGAAGGCTTGGACTGATCCTGCTTTTAAACAA
AGATTGCTAGAAGATCCAGAGACTGTATTAAGGGAGCTAGGATACTATGG
TTTACAGGGTGAGCATATCAGGGTAGTAGAAAATACGGATACGGTACACA
ATGTTGTAGTCTGCACTTTATGTTCATGTTACCCTTGGCCATTGCTTGGT
TTACCGCCTTCATGGTACAAAGAACCTGCTTATAGAGCTCGTGTCGTAAA
AGAGCCGAGACAAGTGTTGAAAGAATTCGGATTAGATCTTCCAGATTCAG
TAGAAATCCGGGTATGGGACAGCAGTTCAGAAATTCGCTTTATGGTATTG
CCGCAAAGACCTGAAGGTACGGAAGGAATGACGGAGGAGGAGCTTGCAAA
ACTTGTTACTCGAGACTCCATGATTGGTGTCGCTAAAATAGAGCCGCTAA
AGTTACGGTAGGTTAGGAGGAAAATAATGAAAAGTTGTGAGAATCAACCT - orf1
AATGAATCATTGCTTGCGAATATGTCTGAAGAAGTCGCACCTCCTAGAAA
AAACGGAGAGTTAGAATTCCAAGAGCCTTGGGAAAGACGCTCTTTTGGCA
TGACTCTTGCTTTGTACGAAGAAAAGCTGTATAGCTCTTGGGAGGATTTT
CGATCCCGCTTGATTGAGGAGATCAAGGGGTGGGAGACCGCGAAACAGAA
GGAGAATTCTGACTGGAACTACTATGAGCATTGGCTGGCCGCCTTGGAAC
GACTAGTAGTGGAAACAGGAATGTTAAATTAAGCGTGATGTCGAC
SalI

FIG. 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06888

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 1/00, 1/20, 9/80, 9/88; C12P 13/02
US CL : 435/129, 228, 232, 252.5, 832; 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/129, 228, 232, 252.5, 832; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/Bioscience cluster
search terms: nitrile hydratase or nitrilase, bacillus, sp. 13, cobalt, amidase or acylamidase or acylase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAINEY, F.A. et al. The phylogenetic diversity of thermophilic members of the genus Bacillus as revealed by 16S rDNA analysis. FEMS Microbiol. Lett. 1994, Vol. 115, pages 205-211, especially page 206.	8-14, 32
X, P	PEREIRA, R. A. et al. A Novel thermostable nitrile hydratase. Extremophiles. February 1998, Vol. 2, pages 347-357, see entire document.	8-30, 32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
07 JUNE 1999	15 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D. Lawrence for ROBERT A. WAX Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06888

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3-7, 31 AND 33
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

this application is not in compliance with the sequence rules. The claims listed require a sequence search.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.